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THE AUSTRALIAN JOURNAL OF EXPERIMENTAL BIOLOGY AND MEDICAL SCIENCE

EDITOR-IN-CHIEF
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VOL. XXXIV

PUBLISHED BY THE UNIVERSITY OF ADELAIDE

PRINTED AT THE GRIFFIN PRESS, MARLBOROUGH PLACE, ADELAIDE
1956.

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COMPARATIVE STUDY OF RECOMBINANTS OF DIFFERENT TYPES OF INFLUENZA A VIRUS WITH THE STRAIN WSE

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(Accepted for publication 3rd November, 1955.)

Previous reports from this laboratory on recombination between influenza virus strains in the allantoic cells of the chick embryo have dealt for the most part with recombination between the classical type A strains WSE and Melbourne (MEL) (Burnet and Lind, 1952) and between their various derivatives (Lind and Burnet, 1953; Lind and Burnet, 1954). Recombination between the influenza B strains has also been described by Perry, van den Ende and Burnet (1954). Recently a study has been made of recombination between WSE and the A prime strain CAM (Burnet and Lind, 1955). The forms obtained were found to be of the same general character as the M+ and WS— recombinants derived from WSE and MEL. However, there was evidence that for recombination to take place between these two viruses, the genetic structure of WSE had to provide a different type of linkage group from that concerned in its recombination with MEL.

The work to be reported here is an extension of this study and was planned primarily as an attempt to obtain recombinants between WSE and all the chief types of influenza A from Shope's swine influenza virus, SW15, isolated in 1931 to the A prime strain Persia, isolated in 1952. The results suggest that, as might be expected, recombination succeeds more readily in strains that are closer together in the temporal (and serological) sequence.

In view of the fact that with each strain there were minor differences in the results and in the methods used, it has seemed best to give details of three sets of recombination experiments, those in which the strains Swine, SR-CAM and Persia interacted with WSE. In the final section the results are compared with those obtained in previous work with MEL and CAM strains and in the present series with several other recent A strains.

MATERIALS AND METHODS.

Virus strains.

WSE. A substrain of the classical WS influenza type A strain (Andrewes, Laidlaw and Smith, 1934) which was derived by adaptation to growth on the chorioallantois.

[1]This work was aided by a grant from the National Health and Medical Research Council, Canberra.

Swine (SW). Shope's strain Swine 15 (Shope, 1931) received through Dr. C. H. Andrewes in 1936.

Serum resistant CAM (SR-CAM). Derived from the influenza A prime strain CAM (Burnet and Anderson, 1947) by serial allantoic passage in the presence of CAM antiserum (Burnet and Lind, 1954a).

Ocean Island (Oc. Is.). An influenza A prime strain (Isaacs, Edney, Donnelley and Ingram, 1950).

CHO. An influenza A prime strain (Anderson, Donnelley, French, Kalra and White, 1953).

Persia (PER). An influenza A prime strain A/Persian Gulf/2/52 (Donald and Isaacs, 1954).

For use in experimental work stocks of virus maintained in 50 p.c. glycerol at −12° C. were given a further allantoic passage at a dilution of 10^{-4} and harvested after 42 hours' incubation at 35° C. All viruses were used on the day of harvest.

Diluents.

Saline. 9 gm. of NaCl made up to 1,000 ml. with distilled water.

Normal horse serum saline (NHSS.) Saline containing 10 p.c. normal inactivated horse serum.

Ringer's solution containing glucose and antibiotics (GPSR). Ringer's solution (NaCl 0·85 gm. KCl 0·025 gm. and $CaCl_2$ 0·03 gm. made up to 100 ml. with distilled water) to which glucose 0·1 p.c., penicillin (20 units/ml.) and streptomycin (70 μg. per ml.) are added.

Titration of haemagglutinin (HA). Serial twofold dilutions of virus were prepared in 0·25 ml. volumes of saline. An equal quantity of a 1 p.c. suspension of fowl erythrocytes was added to each tube. After 45 minutes' settling at room temperature the end point was read by determining the dilution giving a standard "+" pattern of partial agglutination. The reciprocal of this dilution was taken as the titre of the virus.

Neutralization of virus haemagglutinin by antiserum. Throughout most of this work the serological type of a virus was determined by adding a dilution containing 5 agglutinating doses (A.D.) to an equal amount (0·25 ml.) of a 1/200 dilution of each of the relevant antisera which had previously been treated with potassium periodate to destroy non-specific haemagglutinin inhibitor (Burnet and Lind, 1954b). This was followed by the addition of 0·25 ml. of 1 p.c. fowl red cells. The presence or absence of haemagglutination could be determined after the cells had settled for 15–20 minutes at room temperature.

The serological character is represented according to the convention used previously—MEL represented by A, WSE by a, and the other types by A with a suitable superscript, A_{SW} for SW, A_{CR} for SR-CAM, A_{CH} for CHO and A_P for Persia.

Determination of "Indicator" status. Virus allantoic fluids were mixed with an equal quantity of 0·06 M veronal buffer pH 7·0 and heated at 52° C. for 30 minutes (Burnet and Lind, 1955). The residual HA was determined and 0·25 ml. volumes of a dilution containing 5 A.D. were added to equal amounts of serial tenfold dilutions of various mucoids. After 30 minutes at room temperature, 0·25 ml. of 1 p.c. fowl red cells was added. The end point of HA inhibition was read after a further hour at room temperature. The mucoid inhibitor used throughout this work was ovomucin (Gottschalk and Lind, 1949) and occasionally sheep salivary gland mucoid (McCrea, 1953) was included as well. Those viruses whose HA was inhibited by ovomucin under these conditions are referred to as "indicator" viruses, the others as "non-indicators," the former being represented by c and the "non-indicators" by C. The reactions with sheep mucoid are similarly represented by d and D.

Determination of chick embryo pathogenicity. Virus fluids were diluted in NHSS to a titre of 1 A.D. and 0·05 ml. inoculated on to the chorioallantoic membrane (CA) of 12-day chick embryos. The eggs were incubated for 3 days when they were candled, all dead

embryos examined and the remainder returned to the incubation for a further 24 hours before being opened and examined for foci on the CA membrane, for death of the embryo and the presence of haemorrhagic lesions. Death of the embryo on the 3rd day of incubation scored 2, on the 4th day, 1; lesions in brain, muscles and lungs were graded 0–3 and in heart and spleen 0–2, making a maximal possible score of 15. The figures shown in the Tables represent the average scores of groups of 4–8 embryos. When the score was less than 3·0 the strain was regarded as non-pathogenic and indicated by E; when haemorrhagic lesions were present giving a score of 3·0 or greater, the strain was considered pathogenic and indicated by e.

Determination of mouse lung pathogenicity. Virus fluids were diluted in NHSS to a titre of 1 A.D. and 0·05 ml. amounts inoculated intranasally into mice under light ether/chloroform anaesthesia.

The mice were killed 7 days after inoculation and the lung lesions were scored 1–3·9 for living mice according to the degree of consolidation. Death on the 5th day or earlier scored 5, on the 6th day 4·5 and on the 7th day 4. Virus strains which, when inoculated under these conditions, caused consolidation of the lungs with or without causing death of the mouse were designated f. Those which produced no lesions were designated F.

It might be pointed out here that in the original definition of the Ff character for the MEL/WSE system (Burnet and Lind, 1952), f represented fatal pneumonia and F represented a non-fatal infection in which consolidation occurred but did not involve more than 50 p.c. of the lung area. Under the circumstances it is difficult to avoid confusion with the nomenclature previously used. As a practical compromise, it has seemed best to express the MEL character as (F), the brackets differentiating it from the quality F which signifies that no lesions are produced.

Table 1 summarizes the characteristics of the virus strains used in this work in terms of the above markers.

TABLE 1.

Characteristics of Influenza A strains used or referred to in this work.

Strain	Year of isolation	Serology	Indicator status		Pathogenicity for	
			Ovomucin	Sheep mucoid	Chick embryo	Mouse lung
SW	1931	Asw	?[2]	D	E	f
WSE	1933	a	c	d	e	f
MEL	1935	A	C	D	E	(F)
SR-CAM[1]	1946	Acr	C	D	E	F
CHO[1]	1950	Ach	C	D	E	F
PER[1]	1952	Ap	c	D	E	F

The significance of the symbols is explained in the text under Methods.

[1] Allantoic fluids of PER showed large numbers of filaments. SR-CAM and CHO strains showed a smaller proportion.

[2] Results irregular.

Recombination experiments. These were carried out by the method previously described (Burnet and Lind, 1954c). Freshly harvested virus fluids were diluted in GPSR to 20 A.D. titre. These were mixed in the proportions required and 5 ml. of each mixture inoculated into pairs of de-embryonated eggs (DEE) prepared from chick embryos which had had 14–16 days' preliminary incubation. The eggs were attached to a rotating disc set in a 35° C. incubator as described earlier. After 1 hour's incubation the inoculum was removed and

the DEE washed out thoroughly with three changes of Ringer's solution before being refilled with 5 ml. of GPSR and returned to the incubator. The fluid was harvested 7 hours after infection, distributed in ampoules for storage in a CO_2 ice box and subsequently examined for the presence of recombinants.

Determination of double neutralization. The 7-hour harvests from recombination experiments were titrated for HA titre using as diluents saline and a 1/200 dilution of periodate-treated antiserum against each of the virus strains used (Burnet and Lind, 1954b). The sum of the titres in the two antisera was expressed as a percentage of the titre in saline. It was found that with the controls, or with artificial mixtures of the two viruses concerned, the sum of the titres in the two antisera was 50 p.c. or more of the saline titres, so that a percentage of less than 50 was indicative of neutralization by both antisera.

Limit dilution (L.D.) titration. Serial tenfold dilutions of virus were prepared in NHSS and 0·05 ml. volumes inoculated allantoically into groups of 11- and 12-day-old chick embryos. After 2 days' incubation at 35° C. samples of allantoic fluid from each egg were tested for the presence of virus HA. Allantoic fluids were harvested from eggs giving positive reactions and negative eggs returned for a further day's incubation before retesting. With strain Persia, which took a longer time to reach maximum titre than the other strains, the eggs were incubated for 3 days without the 2-day sample test. Positive fluids derived from eggs inoculated with the highest infective dilution of virus were used for further work. These are referred to as limit dilution or L.D. fluids.

Intravenous inoculation of chick embryos. This was carried out according to the method of Beveridge and Burnet (1946).

EXPERIMENTAL.

Recombination between WSE and SW (Swine 15) viruses.

In earlier work on recombination between the mouse neurotropic WS strain, NWS (Stuart-Harris, 1939), and non-neurotropic SW virus typical recombinants were obtained which were serologically SW, but had a high pathogenicity for mice by intracerebral injection (Burnet and Lind, 1951). It was, therefore, expected that recombination between WSE and SW would also be possible.

The marker characters that were used to differentiate SW from WSE were those mentioned under Methods and shown in Table 1. The following notes will expand certain points. The indicator status of SW is ill-defined, simple heating in veronal buffer at 52° C. increasing susceptibility to inhibition by ovomucin to a variable titre. The recombinant SW+ strains were consistently converted to indicator, but the stock strain results were too irregular to allow much use to be made of this character as a marker. Our stock SW produces easily visible small foci on the chorioallantoic membrane, but rarely kills the embryo and never produces haemorrhagic lesions. Intravenous inoculation of 1 A.D. killed chick embryos in 3 days without haemorrhagic lesions.

In the experiments chick pathogenicity was the most important feature characterizing the recombinant strains, but the indicator reactions were also tested.

In the first recombination experiment to be undertaken with WSE and SW viruses, both were diluted to the 20 A.D. titre level, mixed in the proportions

by volume 5:1, 1:1 and 1:5 and the mixtures inoculated into pairs of DEE. All 7-hour harvests from doubly infected eggs showed the double neutralization phenomenon.

Each of the 7-hour harvests was serially diluted in anti-WS serum 1/20 and inoculated into 12-day chick embryos by the chorioallantoic route. Inoculation of harvests from the SW1:WSE5 and the SW5:WSE1 groups of DEE caused the death of a number of embryos. In most of the HA titre of saline extracts of the organs was high enough to allow a direct serological test. Virus of WS serological type was present in both hearts and lungs of several of these. From these allantoic inoculation to limit infecting dilution allowed the isolation of pure clones of the virus concerned. These were serologically SW and on chorioallantoic inoculation caused death of embryos with the pathological changes described below. This recombinant virus was named SW+. Limit dilution allantoic passage of SW+ was carried out for 2 successive passages, the harvests from each passage being tested for serology, indicator status and chick embryo pathogenicity. The characteristics of the strain are shown in Table 2.

TABLE 2.

Characteristics of SW+virus.

Harvest from DEE inoculated with	Original lung suspension	1st L.D.[1]	2nd L.D.	Average lesion scores of embryos inoculated chorioallantoically	
				Dilution of inoculum	Score
SW5 : WSE1	54125	54479[2]		10^{-4}	3·5
				1 A.D.	3·0
		54480		1 A.D.	1·0
			54644	1 A.D.	5·5
			54645	1 A.D.	4·5
SW1 : WSE5	54147	54197		10^{-4}	8·0
				1 A.D.	5·5
			54332	1 A.D.	5·3
Swine Stock				1 A.D.	0·0
WSE Stock				1 A.D.	8·0

All SW+ fluids shown in this Table were of Swine serological type and were converted to indicator on heating at 52° C. for 30 minutes at pH 7.

[1] For the 1st limit dilution titration serial dilutions of virus were prepared in anti-WSE serum 1/20.

[2] Fluid used for passage.

The lesions in chick embryos dead as a result of chorioallantoic inoculation of SW+ differed considerably from those obtained when WSE virus was inoculated by the same route. Cerebral haemorrhage was not seen, but pin-point haemorrhages were present in the muscles and in almost all cases there was some degree of lung consolidation and discrete white lesions were present in the

spleen. An unusual feature was the occurrence of haemorrhagic lesions of varying degrees of severity in the hearts of more than 50 p.c. of the affected embryos. In Table 3 the distribution of lesions in embryos dying after chorioallantoic infection with WSE and SW+ viruses are compared.

TABLE 3.

Comparison of distribution of lesions in embryos inoculated chorioallantoically with SW+ and WSE viruses.

Infecting virus	No. of embryos		Brain (3)[1]	Muscles (3)	Lungs (3)	Heart (2)	Spleen (2)
SW+	21	Total score[2]	1	35	37	26	39
		Average score	0·05	1·7	1·8	1·2	1·9
		No. positive / No. inoculated	1/21	21/21	20/21	19/21	21/21
WSE	13	Total score	30	29	25	4	18
		Average score	2·3	2·2	1·9	0·3	1·4
		No. positive / No. inoculated	13/13	13/13	12/13	3/13	13/13

[1] Numbers shown in brackets under the name of each organ represent score for maximal lesions in that organ. Scores graded 0, 1, 2, 3 or 0, 1, 2.

[2] Total score represents sum of the scores for lesions in any one organ of all the embryos examined.

In other experiments, 3 additional strains of SW+ type were obtained, differing in no significant respect from those shown in Table 2.

Recombination between WSE and SR-CAM.

In previous work a study was made of recombination between various derivatives of the 1946 A strain CAM (Burnet and Lind, 1954a). In the course of that work a serum-resistant strain SR-CAM was prepared by repeated growth in anti-CAM serum. This strain was hardly affected by anti-CAM serum, but was neutralized both *in ovo* and in anti-HA titrations by a homologous rabbit antiserum. Mainly because it was thought that a recombinant with the unusual serological character of SR-CAM and the pathogenicity of WSE would be a valuable reagent for further work, recombination experiments were undertaken.

The method was modified by using the first 7-hour fluid as one of the components of a second DEE recombination experiment with a fresh amount of SR-CAM. Two 7-hour harvests were thus available, the first (I) from an initial mixture of equal amounts (20 A.D.) of WSE and SR-CAM and the second (II) from equal amounts of harvest I and SR-CAM. Both harvests showed double neutralization *in vitro*. Each fluid was serially diluted in anti-WS/20 and inoculated onto the chorioallantois.

In each case several embryos died with haemorrhagic lesions and lung and heart suspensions contained sufficient virus to allow its characterization by anti-haemagglutinin tests as of SR-CAM serology. One lung suspension, 55866, was neutralized by both antisera. Three such lung emulsions were used as starting points for a series of L.D. passages and at each passage L.D. fluids were tested for serology, indicator status and pathogenicity for chick embryos by chorio-allantoic inoculation and for mice by the intranasal route. The results given in Table 4 show three fairly consistent patterns. All are serologically SR-CAM and are converted to indicator on heating under standard conditions. In the first series pathogenicity for both chick embryo and mouse is either absent or very low. The series derived from 56018 is highly pathogenic for chick embryos, but not for mice. That from 56019 has in addition a moderate and consistent virulence for mice.

Although the strain from lung 55866 did not kill the chick embryo when

TABLE 4.

Characteristics of SR-CAM + recombinants isolated by chorioallantoic inoculation of harvests I and II from WSE/SR-CAM recombination experiments.

Harvest No.	Original lung suspension	1st L.D.[1]	2nd L.D.	3rd L.D.	Characteristics of virus			
					Serology	Indicator status	Chick embryo pathogenicity	Mouse lung pathogenicity
	55866				Neutralized by anti-WS and anti-SR-CAM sera.			
I		56409			SR-CAM	Indicator	0	<0·1
		56410[2]			SR-CAM	Indicator	0·8	0·2
			57057		SR-CAM	Indicator	0	0
	56018				SR-CAM			
		56297[2]			SR-CAM	Indicator	3·0	0
		56301			SR-CAM	Indicator	4·3	0
			56658		SR-CAM	Indicator	6·0	0
II				56834	SR-CAM	Indicator	5·0	0
	56019				SR-CAM	Indicator		
		56314			SR-CAM	Indicator	3·5	1·4
		56315[2]			SR-CAM	Indicator	6·0	1·1
			56670		SR-CAM	Indicator	5·0	0·4
				56842	SR-CAM	Indicator	3·3	0·6
WSE Stock					WSE	Indicator	8	4·5
SR-CAM Stock					SR-CAM	Non-indicator	0	0

[1] For the 1st limit dilution titration serial dilutions of virus were prepared in anti-WSE serum 1/20.

[2] Fluid used for passage.

inoculated onto the chorioallantois, it produced small clearly defined pocks on the chorioallantoic membrane. An attempt was made to enhance the chick embryo pathogenicity of this strain by serial passage of chorioallantoic membrane emulsion. Allantoic fluid 56410 (see Table 4) was diluted to 1 A.D. titre and inoculated onto the chorioallantois of 12-day embryos. After 3 days' incubation, one egg was selected at random, the chorioallantoic membrane removed, checked for the presence of pocks, ground with alundum and suspended in 1 ml. of saline. The supernatant after light centrifugation was used without further dilution to inoculate a further group of 12-day embryos. The remaining eggs in the group from which the membrane was obtained were opened after 4 days' incubation and examined for death of the embryo and lesions. Serial transfer was continued for 4 passages. Embryos from the 1st CA passage of 56410 gave an average lesion score of 1·6 which is below the level of significance. The next 3 passages gave negative results.

A similar attempt was made to enhance the mouse lung pathogenicity of the two lines represented by the fluids 56410 and 56834 (see Table 4). Both fluids were diluted to 1 A.D. titre and inoculated intranasally into 6 4-week-old mice. After 2 days, 2 mice in each group were sacrificed and bronchial washings obtained by the method of Fazekas (1948). These washings were titrated for haemagglutinin and inoculated into fresh mice either undiluted or, where necessary, diluted to 1 A.D. The remaining 4 mice in the group were killed 7 days after inoculation and the lesion scores of their lungs were determined.

The passage series commenced with fluid 56410 gave an average lesion score, after one mouse lung passage, of 0·27 and a 5th pasasge score of 3·3, and that commenced with 56834 a 1st passage score of 0 and a 5th passage score of 2·1, indicating rapid adaptation to mouse pathogenicity.

Recombination between WSE and Persia (PER) viruses.

The strain PER was included both as a very recent isolation and because of its highly filamentous character (Donald and Isaacs, 1954). This provided another marker difference from WSE, which has an almost exclusively spherical morphology, only an occasional short filament being observed. Since filamentous forms are clearly visible by ordinary dark-field microscopy, this method was used throughout to determine morphology of the virus in experimental fluids. The other characters of Persia are shown in Table 1. It differs from most of the other strains used in being converted to the indicator state by simple heating at 52° C. for 30 minutes at pH 7·0.

For the recombination experiment fresh virus of each strain was diluted to a titre of 20 A.D. and mixtures of these dilutions prepared in which the proportions were WSE1:PER5 and WSE1:PER1. Pairs of DEE were inoculated with the mixtures and other DEE with each virus alone. The 7-hour harvests were tested for double neutralization and analysed for their content of recom-

binants. There was some evidence for double neutralization of the haemagglutinin in harvests from eggs inoculated with the two virus mixtures, but these were on the threshold of significance.

The harvests from the DEE inoculated with both PERS and WSE were diluted to 10^{-4}, 10^{-3} and 10^{-2} in anti-WS serum 1/20 and inoculated intravenously into 12-day embryos. Those which received the WSE1: PER1 harvest gave negative results. Only one egg from the 10^{-2} group died and the embryo showed no lesions nor was there *in vitro* evidence of virus in either heart or lungs.

Of the eggs which received WSE1:PER5 harvest all survived in the groups given the 10^{-3} and 10^{-4} inocula, but in the 10^{-2} group 2 embryos died with haemorrhagic lesions in muscles and lungs. Suspensions of lungs and hearts from these embryos were tested *in vitro* and one, 57788, found to contain virus of PER serological type, the other, virus of WS serology. A pool of the heart and lung suspensions from 57788 was diluted to 1 A.D. and inoculated onto the chorioallantois both alone and in the presence of anti-WS serum. A second sample was titrated to limit infecting dilution by the allantoic route.

From both these lines extensive series of L.D. passages were made involving several sublines. In all 15 L.D. fluids were tested for chick embryo pathogenicity and 13 for mouse pathogenicity. The most striking feature was the differentiation of clones, in apparently random fashion, into those with moderate or high virulence for chick embryos and those with no virulence. These results are included in Table 5.

TABLE 5.

Analysis of virulence for chick embryo and mouse lung of derivatives of PER+ and of stock PER strains.

Source	Clones with embryo pathogenicity					Clones with mouse pathogenicity				
	Total	Score[1]				Total	Score[2]			
		+++	++	+	0		+++	++	+	0
PER+57788	15	5	2	1	7	13	8	5	0	0
PER+59837	10	5	4	1	0	21	0	2	1	18
PER Stock	4	0	0	0	4	4	0	0	0	4

[1] Scoring convention described under Methods
+++ average score >4
++ average score 2·2·4
+ average score 1·2
0 average score <1

[2] Scoring convention described under Methods
+++ average score >3·5
++ average score 1·5-3·5
+ average score 0·1-1·4
0 average score <0·1

All fluids from these lines contained virus of PER serology and microscopic examination of infected allantoic fluids under dark ground illumination showed that only virus of spherical morphology was present. From both lines substrains were obtained which were virulent for both mice and chick embryos. Such strains were termed PER+.

A second analysis was made of the harvest from the DEE inoculated with WSE1:PER5, using the same methods, but inoculating larger numbers of eggs than before. Of the 12 embryos inoculated intravenously with a 10^{-2} dilution in anti-WS serum, 5 died showing lesions of varying degrees of severity and their lungs contained virus of PER serological type. Upon allantoic titration, all lung suspensions yielded PER+ virus of PER serology, of spherical shape and showing a wide range of chick embryo and mouse lung pathogenicity. The results are summarized in Table 6.

TABLE 6.

Characteristics of 5 lines of PER+ virus obtained by recombination between PER and WSE.

Lung Suspension	1st L.D.	2nd L.D.	Characteristics of the virus			
			Serology	Filaments	Chick pathogenicity	Mouse pathogenicity
59296			Persia			
	59826		Persia	—	3·8	3·5
	59829		Persia	—	3·0	3·4
59298						
	59836		Persia	—	3·0	2·5
	59837		Persia	—	4·5	0·8
59301			Persia			
	59731		Persia	—	4·0	2·1
		59875	Persia	—	2·0	2·0
59303			Persia			
	59593		Persia	—	2·4	0·05
		60172	Persia	—	3·0	0
		60173	Persia	—	1·0	0
	59595		Persia		2·0	0
		61368	Persia	—	2·0	0·1 atypical
		61371	Persia	—	0	0·05
59303B						
	59853		Persia	—	3·0	3·7
	59854		Persia	—	1·4	2·1
Persia Stock			Persia	++	0	0
WSE Stock			WS	—	8	4·5

One line represented by fluid 59837, shown in Table 6, was chosen for further study mainly because of its low mouse pathogenicity. A total of 21 fluids was tested for mouse pathogenicity and about half of them for chick embryo pathogenicity as well, with results that are also shown in Table 5, where they can be compared with derivatives of PER+ 57788 and with stock PER

virus. It will be seen that the most usual combination of activities for these PER+ strains is moderate to high pathogenicity for chick embryos without virulence for the mouse lung. Once established, descendants of a given line gave reasonably consistent results (see Table 7).

TABLE 7.

Relation between pathogenicity for the chick embryo of the 1st L.D. fluid and of 2nd L.D. derivatives of PER+ strains 57788 and 59837.

1st L.D. Fluids		Scores for individual 2nd L.D. derivatives					
Score[1]	Number						
0	3	0	0	0	0	+	
+	1	+	++				
++	3	0[2]	+	++	+++	+++	+++
+++	3	+	++	++	+++	+++	+++

[1] See footnote 1 Table 5.
[2] A 3rd L.D. passage gave fluids with scores +++ and +.

(Detailed passage charts from which Tables 5 and 7 were prepared are available in the Institute Library).

One line of PER+ was submitted to serial chorioallantoic passage in an attempt to see whether chick embryo virulence could be raised to the level of WSE. For each passage lungs, usually taken from an embryo dead at 3 days, were emulsified in saline and the supernatant diluted to 1 A.D. in saline and used as inoculum for 4 embryos. Lesions were recorded as described under

TABLE 8.

Results of serial chorioallantoic passage of a. Persia + strain.

C.A. Passage No.	Average lesion score in 4 embryos
1	4·8
2	4·3
3	5·5
4	4·0
5	4·2
6	9·0
7	7·0
8	4·5
9	7·0
10	8·5
11	3·2
12	7·0
L.D. fluids from allantoic titration of 12th C.A. passage material	6·2
	9·2

Methods. After 12 passages the last lung emulsion was titrated to L.D. in the allantoic cavity and two L.D. fluids tested for chick and mouse pathogenicity.

The results in Table 8 show a progressive if somewhat irregular increase in the lesion-producing power on chorioallantoic inoculation. There was no significant alteration in the mouse pathogenicity, the scores being 1·8 and 0·7 for the final L.D. fluids.

Recombination between CHO and WSE.

Using similar techniques, a chick embryo, 53383, inoculated with the primary fluid from a CHO5/WSE1 experiment (and anti-WS serum), gave haemorrhagic lesions and a CHO type virus was found to be present in the lung emulsion. Two limit dilution passages were made and two fluids from each characterized with results which are shown in Table 9.

TABLE 9.

Characteristics of CHO+ recombinant.

Original lung suspension	1st L.D. passage	2nd L.D. passage	Characteristics of virus		
			Serology	Indicator status	Chick embryo pathogenicity[1]
53383			CHO	Indicator	0, 0, 0, 2*
	53599		CHO	Indicator	0, 0, S, 6*
		53746	CHO	Indicator	S*, S, 0, 0
		53749	CHO	Indicator	0, 0, 0, 0
	53600		CHO	Indicator	S, S, 0, S*
		53758	CHO	Indicator	0, 0, 0, S
		53762	CHO	Indicator	0, 0, 0, 0
WSE Stock			WSE	Indicator	9, 7, 8, 9
CHO Stock			CHO	Non-indicator	0, 0, 0, 0

[1] Results given in terms of lesion scores for individual embryos. S = sick embryo, no lesions.
* CHO+ isolated by allantoic passage of embryo lung emulsion.

Although the effects on the chick embryo were minimal, well-developed small foci developed on the membranes of all eggs inoculated with the CHO+ recombinant by the chorioallantoic route. No lesions were produced on intranasal inoculation of mice.

Similar experiments with the strain Oc.Is. and WSE gave no recombinant forms.

DISCUSSION.

This work has shown that, broadly speaking, recombinants of the same general type as M+ can be obtained by recombination between the strain WSE and any of the available strains of influenza A. The only complete failure was with the strain Oc.Is. The degree to which the characteristic features of WSE,

its convertibility to indicator on heating and its pathogenicity for chick embryos and mice, was present in the recombinants, varied from strain to strain and amongst individual recombinant clones derived from the same experiment.

The essential features of all the well-defined recombinant types which were isolated and purified in the course of this work are shown in Table 10.

TABLE 10.

Characteristics of A+ recombinants obtained from mixed infections of WSE with the influenza A strains shown.

Strain	Fluid No.	Serology	Indicator status	Chick embryo pathogenicity[1]	Mouse lung pathogenicity[1]	Remarks
SW	Stock	SW	variable	−	+ (4)	
SW+	54644 2nd L.D.	SW	indicator	+ (5·5)	+ (4)	3 other similar strains
MEL		MEL	non-indicator	−	+ (1·2)	See Burnet and Lind (1952)
M+		MEL	indicator	+	+ (1·2)	
CAM		CAM	non-indicator	−	−	See Burnet and Lind (1955)
CAM+		CAM	indicator	+	+ (2·3)	
SR-CAM	Stock	SR-CAM	non-indicator	−	−	
SR-CAM+	56834 (3rd L.D.)	SR-CAM	indicator	+ (5·0)	−	
SR-CAM+	56842 (3rd L.D.)	SR-CAM	indicator	+ (3·0)	+ (0·6)	
CHO	Stock	CHO	non-indicator	−	−	
CHO+	53746 (2nd L.D.)	CHO	indicator	±	−	
PER	Stock	PER	indicator	−	−	
PER+	59826 (2nd L.D.)	PER	indicator	+ (3·8)	+ (3·5)	
PER+	60172 (2nd L.D.)	PER	indicator	+ (3·0)	−	

[1] Figures in parentheses represent average scores.

We have not encountered any new phenomena which appear to require interpretations other than those already developed in papers from this laboratory.

Double neutralization of first cycle fluids. Where there was satisfactory serological differentiation between WSE and the other reacting strain, double neutralization of the primary product of interaction could nearly always be demonstrated. Nonspecific inhibition of haemagglutinin by anti-WS serum made work with some of the more recently isolated strains unsatisfactory in this regard. In all probability the use of absorbed antisera would have allowed

us to obtain clearer results, but as the point was not one of primary interest this was not done, nor is more than passing mention made of the results in the body of the paper.

Interchange of linkage groups. Except where the yield of viable recombinants is very high, as in the MEL/WSE and M+/WS— systems, it is difficult or impossible to isolate recombinants without providing a selective environment to eliminate unmodified parent forms. It is easy to arrange this when a chick embryo pathogenic recombinant is being sought. The fluid containing the recombinants is diluted in anti-WS serum, which will eliminate WSE and WS—, and titrated on the chorioallantois or intravenously, to provide a selective environment for the multiplication of chick-pathogenic recombinants.

There are several grades of chick embryo pathogenicity which may be conveniently divided into four types:

(1) Production of well-defined chorionic foci but no invasion of the embryo from the chorioallantois.

(2) Invasion of the embryo with production of minor proliferative lesions and usually death of the embryo.

(3) Invasion with haemorrhagic lesions in various organs and death of the embryo.

(4) Failure to invade embryo but capacity to kill with haemorrhagic lesions when virus inoculated intravenously.

There is also a dosage effect and in all tests virus was diluted to the 1 A.D. level before being tested.

Depending on the nature of the strain being used for recombination with WSE, either chorioallantoic or intravenous inoculation may be the more suitable to obtain chick-pathogenic recombinants. Where the stock strain has no pathogenicity when given intravenously this appears to be the most satisfactory route and gave excellent results in the experiments on PER/WSE recombination.

Where the original strain was not converted to indicator on simple heating all chick pathogenic recombinants showed a change to "indicator" status.

Mouse pathogenicity was sometimes present in the recombinants, sometimes lacking, and when present it was never of the high grade characteristic of WSE.

In line with results obtained with MEL/WSE crosses, the capacity to become an indicator for sheep mucin was not present in any of the recombinants. No evidence of persisting serological hybrids was obtained, all purified strains being unequivocally of one type or the other.

If, for differentiating characters, we use the symbols which were defined under Methods and summarized in Table 1, the results can be expressed as follows:

WSE adf-ce +

SW	$A_{SW}DfcE$	⟶	$A_{SW}Df$-ce
MEL	AD(F)CE	⟶	ADF-ce
CAM	A_{C}DFCE	⟶	A_{C}D-fce
SR-CAM	A_{CR}DFCE	⟶	A_{CR}D-fce, A_{CR}DF-ce
PER	A_{P}DFcE	⟶	A_{P}D-fce, A_{P}D-Fce, A_{P}D-fcE.

Redistribution of virulence characters. As far as they were studied, the results showed a considerable range in the intensity of pathogenic action on mice and chick embryos by the various recombinants. The points of chief interest which emerged in regard to virulence were, however, (a) the qualitative differences in the generalized lesions produced in chick embryos, and (b) the very weak expression of the "transferable" qualities of WSE in the recombinants with the A strains of more recent origin.

A. Each type of recombinant seemed to have its own pattern of attack on the organs of the chick embryo. With SR-CAM+ and CHO+ a relatively small proportion of embryos showed typical haemorrhagic lesions, many surviving for 4 days or dying without visible lesions. Both + strains, however, produced well-developed focal lesions on the chorioallantois, although the original SR-CAM and CHO strains produced minimal opacity or nothing in this situation. Fig. 1 summarizes the average scores for five tissues of the embryo for WSE, SW+, CAM SR+ and PER+. It will be seen that the most characteristic features are the high proportion of brain haemorrhages with WSE and the common production of haemorrhagic lesions in the heart by SW+.

B. Although pathogenicity for mice was present in many recombinant strains of SR-CAM, PER and CHO, it was always very much below that of stock WSE. A similar weak expression of pathogenicity for the chick embryo in the same recombinants has been noted above. As might be expected, the moderate virulence conveyed by recombination to a different serological type could be enhanced by appropriate passage.

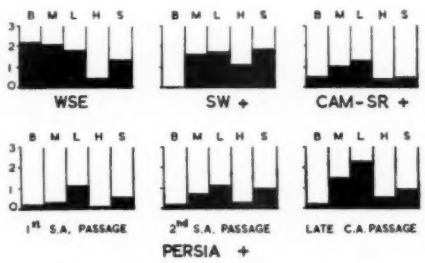

Fig. 1. To show the different patterns of chick embryo lesions with "+" strains.
The average level of intensity is shown for—

B Brain	Maximum score	3
M Muscle	,, ,,	3
L Lung	,, ,,	3
H Heart	,, ,,	2
S Spleen	,, ,,	2

In view of the fact that a review of all the work on genetic interaction from this laboratory is in preparation, no discussion of the general significance of the present results will be attempted.

SUMMARY.

Recombination experiments were carried out between the influenza type A strain WSE, and other type A influenza strains Swine 15, SR-CAM, Ocean Island, CHO and A/Persian Gulf/2/52.

Recombinant virus strains which had acquired various characteristics of WSE, notably convertibility to the indicator state and pathogenicity for chick

embryos and for mouse lung, while retaining the serological type of the other parent strain, were isolated in all experiments except those with Ocean Island virus.

The yield of recombinants in all cases was lower than in previously described experiments and a selective medium was usually needed for their isolation.

The extent to which chick embryo and mouse lung pathogenicity were expressed in the recombinant strains varied from one set of experiments to another and also between individual recombinant clones derived from a single experiment.

These pathogenic activities tended to find weaker expression in the recombinants with the type A strains of more recent origin such as CHO (1950) and Persian Gulf (1952). This weak pathogenicity could be enhanced by appropriate passage.

REFERENCES.

Anderson, S. G., Donnelley, M., French, E. L., Kalra, S. L. and White, J. (1953): Med. J. Austral., ii, p. 44.
Andrewes, C. H., Laidlaw, P. P. and Smith, W. (1934): Lancet, ii, p. 859.
Beveridge, W. I. B. and Burnet, F. M. (1946): Med. Res. Counc. Spec. Rep. Series No. 256, p. 22.
Burnet, F. M. and Anderson, S. G. (1947): Austral. J. exp. Biol., 25, p. 235.
Burnet, F. M. and Lind, P. E. (1951): J. gen. Microbiol., 5, p. 67.
Burnet, F. M. and Lind, P. E. (1952): Austral. J. exp. Biol., 30, p. 469.
Burnet, F. M. and Lind, P. E. (1954a): *Ibid.*, 32, p. 711.
Burnet, F. M. and Lind, P. E. (1954b): *Ibid.*, 32, p. 145.
Burnet, F. M. and Lind, P. E. (1954c): *Ibid.*, 32, p. 153.
Burnet, F. M. and Lind, P. E. (1955): *Ibid.*, 33, p. 281.
Donald, H. B. and Isaacs, A. (1954): J. gen. Microbiol., 11, p. 325.
Fazekas de St. Groth, S. (1948): Austral. J. exp. Biol., 26, p. 29.
Gottschalk, A. and Lind, P. E. (1949): Brit. J. exp. Path., 30, p. 85.
Isaacs, A., Edney, M., Donnelley, M. and Ingram, M. W. (1950): Lancet, i, p. 64.
Lind, P. E. and Burnet, F. M. (1953): Austral. J. exp. Biol., 31, p. 361.
Lind, P. E. and Burnet, F. M. (1954): *Ibid.*, 32, p. 437.
McCrea, J. F. (1953): Biochem. J., 55, p. 132.
Perry, B. T., van den Ende, M. and Burnet, F. M. (1954): Austral. J. exp. Biol., 32, p. 469.
Shope, R. E. (1931): J. exp. Med., 54, p. 373.
Stuart-Harris, C. H. (1939): Lancet, i, p. 497.

ANTIBACTERIAL SUBSTANCES FROM FLOWERING PLANTS

3. ANTIBACTERIAL ACTIVITY OF DRIED AUSTRALIAN PLANTS BY A RAPID DIRECT PLATE TEST

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(Accepted for publication 3rd October, 1955.)

For detection of antibiotic activity in flowering plants, workers have generally used extracts of the plants in water or other solvents. The extracts were tested by the cylinder-plate or cup-plate method against various micro-organisms. Numerous surveys of this kind on seed plants have been summarized by Florey *et al.* (1949). Over 1,000 Australian plants have been surveyed in aqueous extracts in this laboratory (Atkinson and Rainsford, 1946; Atkinson, 1946, 1949) and only about 60 showed activity. By testing alcoholic as well as aqueous extracts of a further 150 dried plants a somewhat higher percentage of positive results was obtained (unpublished results). However, these methods of testing seemed relatively insensitive and their failure to pick up active plants was proved by the results of a new test applied to 124 specimens still available from the group of 150 dried plants. The new test, called here the direct plate test, gave numerous positive results not detected by the extraction method.

Materials and Methods.

The dried plants were provided by the Division of Plant Industry, C.S.I.R.O., and each specimen was received already named and identified by an SN number. The names and SN numbers are given in Table 1 with the results of testing. Most of the plants were collected in Queensland, but a few came from the New Guinea area. Many of the plants were primarily collected for alkaloid tests, but a few were sent on account of their reputed healing or medicinal action on sores and tropical ulcers. For the direct plate tests for antibacterial activity, tryptic digest agar was seeded with the test organism and poured into Petri dishes. A small piece of the required part of the dried plant was placed on the surface of the solid agar. Four or five different specimens were tested in each 10 cm. dish. Many plants were also tested after grinding by placing small piles of the ground-up plant on the agar surface. The dishes were incubated at 37° C. for 18 hours and then examined for zones of inhibition of the growth of the test organism around the plant material. The width of each inhibition zone was measured from the edge of the plant specimen to the point at which growth of the test organism appeared.

The test organisms were *S. typhi* S76, *Staph. aureus* B313 and *Myco. phlei* CSL. In 100 ml. of agar the inoculum was 4 ml. of an overnight broth culture of *S. typhi* or *Staph.*

aureus or 10 ml. of a 24-hours' broth culture of *Myco. phlei*. The test organisms were kept on agar slopes in the refrigerator.

Control tests on the sensitivity of the test plates were always included and consisted at first of pieces of the antibiotic mushroom *Psalliota xanthoderma*, but later included the strongly antibiotic plants found during this work, *Plumbago zeylanica* root, *Rhodomyrtus macrocarpa* fruit and *Lunasia amara* bark.

RESULTS.

The results of the direct plate test on 124 different dried plants are given in Table 1. Where possible the different parts of a plant were tested separately.

The results in Table 1 were derived from several tests on each specimen. The majority were tested whole and after grinding, but no extra positive results were obtained from the ground specimens. Thus grinding was unnecessary and the method recommended for all future work requires only the application of selected portions of the plant specimen to the test agar.

Bacterial contaminants from the plant specimens seldom grew out on the agar. When contaminants appeared the test was repeated and all specimens finally gave readable results. Of the 124 different plants recorded in Table 1, 75 showed inhibitory action in one or more parts against one or more test organisms. Many of these positive reactions, however, were either small zones of partial inhibition (recorded in Table 1 as tr) or very small zones 0·5 to 1 mm. wide of complete inhibition (recorded in Table 1 as ±). These weak reactions were unlikely to indicate the presence of interesting antibiotics. The following 30 plants, however, showed fair to good zones of inhibition (recorded as + or ++ in Table 1) against one or more of the test organisms—*Marsdenia rostrata* (*Asclepiadaceae*) wood, *Heliotropium amplexi* (*Boraginaceae*) whole plant, *Eupatorium adenophorum* (*Compositae*) stem, *Macrozamia hopei* (*Cycadaceae*) nut kernel, *Macrozamia paulo-gulielii* (*Cycadaceae*) nut kernel, *Baloghia lucida* (*Euphorbiaceae*) bark, *Euphorbia plumerioides* (*Euphorbiaceae*) leaves and bark, *Cryptocarya australis* (*Lauraceae*) bark, *Litsea bindornara* (*Lauraceae*) bark, *Petalostigma lanceolatus* (*Leguminosae*) seeds, *Asparagus plumosus* (*Liliaceae*) fruit, *Kreysigia multiflora* (*Liliaceae*) leaves and branchlets, *Strychnos psilosperma* (*Loganiaceae*) leaves, *Doryphora sassafras* (*Monimiaceae*) leaves, *Rhizophora mucronata* (*Rhizophoraceae*) root bark, *Coelospermum reticulatum* (*Rubiaceae*) root, *Guettarda speciosa* (*Rubiaceae*) bark, *Acronychia baueri* (*Rutaceae*) leaves, *Clausena brevistyla* (*Rutaceae*) branchlets, *Evodia xanthoxyloides* (*Rutaceae*) leaves, *Flindersia maculosa* (*Rutaceae*) bark and twigs, *Flindersia* sp. SN5119 (*Rutaceae*) bark, *Geijera parviflora* (*Rutaceae*) bark, *Halfordia kendach* (*Rutaceae*) bark, *Halfordia scleroxyla* (*Rutaceae*) bark, *Micromelum pubescens* (*Rutaceae*) leaves, fruit and branchlet, *Verbascum virgatum* (*Scrophulariaceae*) leaves, stems and bark, *Ternstroemia rhederiana* (*Ternstroemiaceae*) mature fruit seed coat, *Gmelina* sp. SN5286B (*Verbenaceae*) bark.

TABLE 1.

Antibiotic activity of dried plants by the direct plate test.

Plant tested	Part tested	Inhibitory action on		
		S. typhi S76	*Staph. aureus* B313	*Myco. phlei.*
Amaryllidaceae				
Crinum sp. SN5161	root	−	−	−
Crinum sp. SN5163	bulb	−	−	0
Apocynaceae				
Alstonia constricta SN4916	bark	−	++	+++
Alyxia ruscifolia SN5230	leaves	−	−	−
Ervatamia orientalis SN4849	leaves	−	−	−
	bark	±	−	−
Parsonsia straminea SN5146	leaves	−	−	−
	stems	−	−	−
Thevitia nereifolia SN5214	leaves	±	−	tr
Araceae				
Typhonium brownii SN5310G	fruit	−	−	−
Asclepiadaceae				
Asclepius fruticosa SN5219	leaves	−	−	0
	branchlets	tr	−	0
SN5128	leaves	−	−	−
	stems	−	−	−
Marsdenia rostrata SN5198	wood	+	++	++
	bark	±	−	−
Sarcostemma australe SN4825	whole plant	−	−	−
Boraginaceae				
Ehretia acuminata SN5109	leaves	−	−	−
Heliotropium amplexi SN4944	leaves	tr	−	0
	whole plant	+	+	−
	twigs	++	−	0
Casurinaceae				
Casuarina torrulosa SN4909	bark	−	−	−
Celastraceae				
Celastrus diepermus SN5112	root bark	tr	tr	0
	bark	−	−	+++
Chenopodiaceae				
Threlkeldia proceriflora SN4366	twigs	−	−	−
Compositae				
Erechtites atkinsonae SN4929	stem	−	−	0
	leaf	−	−	0
Eupatorium adenophorum SN4920	leaves	±	±	−
	stem	++	tr	−
Sonchus oleraceus SN4878A	whole plant	−	−	−
Vernonia cinerea SN4863	leaves	tr	tr	±
Xanthium pungens SN5227	leaves	−	−	−
	burr and twigs	tr	tr	−
Cucurbitaceae				
Cucumis myriocarpus SN4946	whole plant	tr	tr	−
Cunoniaceae				
Aphanopetalum resinaceum SN5213	leaves	−	−	0
Ceratopetalum apetalum SN5212	bark	−	−	−
Cycadaceae				
Cycas circinalis SN5311	nut outer shell	+	±	−
	nut inner corky layer	−	−	−
	nut kernel	++++	+++	++++
SN5285	growing point, leaf bases and frond	−	−	−

TABLE 1—*continued.*

Plant tested	Part tested	Inhibitory action on		
		S. typhi S76	*Staph. aureus* B313	*Myco. phlei.*
Cycas media SN5310D	leaves	−	−	−
	nut outer layer	−	−	+
	nut hard shell	+	+	tr
	nut kernel	+++	++	++++
Macrozamia hopei SN5310C	leaves	−	−	−
	branchlet	−	−	−
	nut kernel	++(6 hrs.)	++(6 hrs.)	0
	nut shell	−	−	0
Macrozamia paulo-gulielii SN4913	nut kernel	+(6 hrs.)	+(6 hrs.)	−
Dioscoreaceae				
Dioscorea bulbifera var. *rotundata* SN5314C	root	−	−	0
Droseraceae				
Drosera spathulata SN4958	whole plant	−	−	−
Ebenaceae				
Maba rufa SN5310A	leaves	−	−	−
	branchlet	tr	−	−
	fruit	−	−	−
Euphorbiaceae				
Aleurites mollucana SN5314D	fruit outside shell	−	−	−
	fruit kernel	−	−	−
SN5310B	fruit outside shell	−	−	0
	fruit kernel	−	−	0
Baloghia lucida SN5125	bark	+	−	+
Euphorbia plumerioides SN5364	scorched leaves	+	+	+
	leaves	+	+	++
	bark	+	+	++
Excoecaria dallachyana SN5228	bark	tr	±	−
	branchlets	−	−	−
Mallotis angustifolia HN2360	bark	−	−	0
Flacourtaceae				
Homalium circumbinnatum HN2427		±	−	0
Hernandiaceae				
Hernandia bivalvis SN5162	bark and wood	−	−	−
	leaves and branchlet	−	−	0
Hernandia peltata SN5139	bark	−	−	−
Haemodoraceae				
Haemodorum corymbosum SN5142	root	±	±	−
	leaf	−	−	−
Lauraceae				
Cassytha filiformis var. *pseudo-pubescens* SN4853	whole plant	−	−	−
Cryptocarya angulata SN4854	bark	−	±	±
SN5182	bark	−	±	±
Cryptocarya australis SN5118	bark	−	−	+
Cryptocarya corrugata SN4858	bark	−	−	−
Cryptocarya hypospodia SN4875	bark	+	−	−
	leaves	±	−	−
Cryptocarya mackinnoniana LSS4606	bark	−	−	0
Cryptocarya obovata SN4727	bark	−	−	±

TABLE 1—*continued.*

Plant tested	Part tested	Inhibitory action on		
		S. typhi S76	*Staph. aureus* B313	*Myco. phlei.*
Cryptocarya pleurosperma				
SN4527	leaves	0	0	–
SN4548	bark	tr	tr	0
	leaves	–	–	–
SN4868	bark	0	0	+
Cryptocarya sp. *aff australis*	bark and a few leaves			
SN5087		–	–	0
Litsea bindornara 166	bark	+	–	0
Lecithydaceae				
Barringtonia acutangula SN4948	leaves	–	–	0
	twigs	–	–	0
Leguminosae				
Caesalpinia crista 177	seeds	–	tr	0
Cassia alata SN5310E	leaves	–	tr	tr
	branchlet	–	–	tr
Crotalaria agatiflora SN5150	stem	–	–	–
Crotalaria mitchelli SN5126	stem	–	–	–
Crotalaria sericea SN5215	pods	tr	–	tr
Crotalaria striata SN4947	twigs	–	–	–
Cynometra ramiflora var. *bijuga*	bark	–	–	–
SN4904	leaves	–	–	–
	twigs	–	–	0
Erythrina vespertilio SN5127	leaves	–	–	–
	bark	–	–	–
Erythrina phlebocarpa LSS4857	seeds	–	–	–
Gastrolobium grandiflorum				
SN5131	leaves	–	–	–
SN5238	leaves	–	–	–
Isotropis atropurpurea SN5130	leaves	–	–	–
	stems	–	–	–
Petalostigma lanceolatus 173	seeds	±	+	–
Phaseolus lathyroides 45	pods	–	–	–
Pongamia pinnata SN5141	seeds	–	–	–
Robinea pseudoacacia Can. 10832	bark	–	–	–
Swainsonia galegifolia SN4927	whole plant	0	0	–
	leaf	–	–	0
	stem	–	–	0
	pod	–	–	0
Tephrosia grandiflora SN5149	stem	–	–	0
	leaves	–	–	0
	root	–	–	0
	whole plant	0	0	tr
Vicia angustifolia (fruiting)	pods, leaves and twigs	–	–	0
Liliaceae				
Asparagus plumosus SN4644	fruit	++	–	0
	wood	–	–	0
Kreysigia multiflora SN4917	leaves and branchlet	++	+	+
Lobeliaceae				
Lobelia concolor SN5175	whole plant	–	–	–
Loganiaceae				
Strychnos bancroftiana 150	bark	tr	–	0
Strychnos psilosperma SN4891	leaves	++	+	++
	branchlet	±	–	–

TABLE 1—*continued.*

Plant tested	Part tested	Inhibitory action on		
		S. typhi S76	*Staph. aureus* B313	*Myco. phlei.*
Loranthaceae				
Loranthus sp. SN4925	leaves	–	–	tr
	branchlet	–	–	tr
Menispermaceae				
Stephania aculeata 93	twigs	–	–	–
	leaves	±	–	–
Monimiaceae				
Daphnandra sp. SN4971	bark	–	–	0
Doryphora sassafras SN4433	leaves	–	–	++
	twigs	–	–	0
Moraceae				
Cudrania javanensis SN4882	leaves and stems	–	tr	–
Mysinaceae				
Maesa dependens SN4864	twigs	–	–	tr
Myrtaceae				
Rhodomyrtus macrocarpa SN5228	mature fruit	++++	+++	++++
Palmae				
Areca catechu (betel-nut) SN5135	nut husk	–	–	0
	nut kernel	–	–	0
Phytolaccaceae				
Phytolacca dioica SN4914	leaves	–	–	–
	stem	–	–	–
	berries	tr	tr	+++
Plumbaginaceae				
Plumbago zeylanica SN4911	root	+	++++	++++
Ranunculaceae				
Clematis glycinoides SN5310F	whole plant	++	–	–
Rhamnaceae				
Colubrina asiatica SN4879	leaves and stems	tr	–	–
Emmenosperma alphitonioides SN4980	bark	–	–	0
Rhizophoraceae				
Rhizophora mucronata SN4905	root bark outer layer	–	–	–
	root bark inner layer	++	+	+
Rubiaceae				
Coelospermum reticulatum	leaves and twigs	–	–	–
SN5314A	root	+	++	+
SN5237	root bark	±	++	+
Guettarda speciosa 176	bark	+	+	–
Morinda citrifolia SN5448	leaf	–	–	–
	stem	–	–	–
Neonaucina gordoniana LSS4875	bark	tr	–	tr
Nauclea orientalis SN4852	bark	–	–	–
SN4524	bark	–	–	–
Rutaceae				
Achronychia acidula SN4908	leaves	–	–	–
	bark	+	–	–
Achronychia baueri SN4646	leaves	tr	±	+
	bark	tr	±	±
SN4918	leaves	tr	±	0
Acronychia laevis 159	bark	±	±	0
Acronychia sp. LSS4673	bark	tr	tr	0

TABLE 1—*continued.*

Plant tested	Part tested	Inhibitory action on		
		S. typhi S76	*Staph. aureus* B313	*Myco. phlei.*
Clausena brevistyla SN4907	wood	−	tr	tr
	leaves	tr	−	0
	branchlets	+	−	0
	bark	−	−	−
Eremocitrus glauca SN4945	bark	±	−	tr
	leaves	tr	tr	−
Evodia xanthoxyloides SN4892	leaves	tr	++	++
	twigs	tr	−	0
SN4967	leaves	tr	++	++
	stem	tr	tr	0
Flindersia bennettiana SN4921	leaves	tr	−	0
Flindersia maculosa SN4942	bark	±	±	+
	twigs	+	+	0
Flindersia oxyleyana SN4922	leaves and branchlets	++	+++	0
Flindersia xanthoxyla SN5505	leaves and branchlets	++	+++	++++
Flindersia sp. SN5119	leaves	−	−	−
	bark	++	+	++
	branchlets	−	−	−
Geijera parviflora 157	bark	±	±	++
Glycosmis pentaphylla SN4897	leaves	−	−	−
	stems	−	−	−
Halfordia kendach SN5216	bark	−	−	+
Halfordia scleroxyla SN5181	bark	−	−	+
Lunasia amara SN4510	bark	++	++++	++++
Micromelum pubescens SN5096	leaves, fruit and branchlet	−	−	++
Sapindaceae				
Atalaya hemiglauca SN4941	leaves	−	−	−
Scrophulariaceae				
Verbascum virgatum SN5231	leaves and stems	++	−	−
	bark	++	−	−
Simarubaceae				
Picrasma javanica SN5071	bark	−	−	−
Solanaceae				
Cestrum parqui SN4837	leaves, stems and flowers	−	−	−
Stackhousiaceae				
Stackhousia sp. 174	whole plant	tr	tr	0
Symplocaceae				
Symplocos stawelli SN4867	leaves	±	−	0
175	bark	−	−	0
Ternstromiaceae				
Ternstroemia rhederiana SN5286A	bark	−	−	0
	mature fruit seed coat	++	+	−
	outside shell	±	tr	−
	seed	−	−	−
Verbenaceae				
Gmelina sp. SN5286B	bark	±	±	+
Premna corymbosa SN5320	leaves	tr	−	tr
Verbena venosa SN4837A	whole plant	−	−	−
Vitaceae				
Ampelocissus acetosa SN5314E	leaves	−	tr	0
	stems	−	−	0

TABLE 1—*continued.*

Plant tested	Part tested	Inhibitory action on		
		S. typhi S76	*Staph. aureus* B313	*Myco. phlei.*
Zingiberaceae *Tapeinocheilos queenslandiana* LSS4887	stems	–	tr	–
Zygophyllaceae *Zygophyllum apiculatum* SN4943	leaves and stems	–	–	–

– = no inhibition detected.
tr = partial inhibition up to 2 mm.
± = zone of inhibition 0·5-1 mm.
\+ = zone of inhibition 1-3 mm.
++ = zone of inhibition 3-5 mm.
+++ = zone of inhibition 5-7 mm.
++++ = zone of inhibition over 7 mm.
0 = not tested.

The best of these 30 plants were *Marsdenia rostrata, Euphorbia plumerioides, Kreysigia multiflora, Strychnos psilosperma, Rhizophora mucronata, Coelospermum reticulatum* and *Flindersia* sp. SN5119, all of which gave a + or ++ inhibition with all three test organisms. *Evodia xanthoxyloides* also was effective against all three test organisms but gave only weak (tr) inhibition with *S. typhi.* Four plants showed + or ++ inhibition with two test organisms. These were *Baloghia lucida, Guettarda speciosa, Flindersia maculosa* and *Ternstroemia rhederiana.* All these plants, showing moderate and reasonably wide activity, should be worthy of further investigation.

A small group of plants were very active, producing zones of inhibition larger than 5 mm. (+++ and ++++ reactions in Table 1) against one or more of the test organisms. The best of these were the following plants, active against all three test organisms—*Cycas circinalis* (*Cycadaceae*) nut kernel, *Cycas media* (*Cycadaceae*) nut kernel, *Rhodomyrtus macrocarpa* (*Myrtaceae*) fruit, *Plumbago zeylanica* (*Plumbaginaceae*) root, *Flindersia xanthoxyla,* also known as *Flindersia oxyleyana,* (*Rutaceae*) leaves and branchlets and *Lunasia amara* (*Rutaceae*) bark. Others in this very active group affected one or two of the test organisms. Thus *Alstonia constricta* (*Apocynaceae*) bark, inhibited *Staph. aureus* and *Myco. phlei, Celastrus dispermus* (*Celastraceae*) bark and *Phytolacca dioica* (*Phytolaccaceae*) berries both affected only *Myco. phlei.* This group of very active plants might provide interesting antibiotics and should be further investigated.

Thirty-seven plants showed no activity against any of the three test organisms, and 12 plants showed no activity against *Staph. aureus* or *S. typhi* and were not tested with *Myco. phlei.* Fourteen of these belonged to the *Legu-*

minosae, a family in which antibacterial activity was seldom found. Out of 18 members tested only four showed any activity which was only partial or very weak.

The family *Rutaceae*, a good alkaloidal family (Webb, 1949, 1952), was also a good antibiotic family. Of 18 members tested, only one, *Glycosmis pentaphylla*, failed to show any activity. In this family occurred two of the most active plants, *Flindersia xanthoxyla* or *oxyleyana* and *Lunasia amara*, and three of the moderately active plants, *Evodia xanthoxyloides*, *Flindersia* sp. SN5119 and *Geijera parviflora*. Further representatives of this family should be worth testing.

DISCUSSION.

The direct plate method of testing was superior to the extraction method as it gave more positive results and was easily and rapidly carried out. No real difficulty was encountered from bacterial contaminants. Occasionally contaminants grew out from some plant specimens upon which tests were then repeated and readable results were usually obtained. In fact, none of the plants tested failed to give readable results.

About 60 p.c. of the plants tested were active in some degree against one or more of the test organisms. Many of these reactions were, however, weak. About 25 p.c. (30 out of 124) of the plants were moderately active against one or more of the test organisms and about 7 p.c. (9 out of 124) were very active against one or more of the test organisms. All these plants should be worth investigating to characterize the antibiotic substances responsible for their activity. In the very active group, the root of *Plumbago zeylanica* owed its activity to plumbagin, a naphthoquinone with strong antibacterial action, and the antibacterial substances of *Rhodomyrtus macrocarpa* fruit, *Flindersia xanthoxyla* leaves and branchlets and *Lunasia amara* bark are under investigation.

The most active family in these tests was *Rutaceae* and the least active was *Leguminosae*. These two families were represented by only three specimens in the collected results of antibiotic survey plants given by Florey *et al.* (1949). All three showed activity. None of the plants tested here were recorded by Florey *et al.* (1949).

The plant specimens used in the present tests were all dried and some were about 3 years old. The antibiotics of these plants must therefore be stable substances.

SUMMARY.

By direct testing on seeded agar (direct plate test) 75 out of 124 dried Australian plants showed antibacterial activity against one or more of the test

organisms, *Staph. aureus* B313, *S. typhi* S76 or *Myco. phlei* CSL. The most active family was *Rutaceae* and the least active family was *Leguminosae*. Strong antibacterial activity was detected in the nut kernels of *Cycas circinalis* and *Cycas media* (*Cycadaceae*), the root of *Plumbago zeylanica* (*Plumbaginaceae*), the fruit of *Rhodomyrtus macrocarpa* (*Myrtaceae*), the leaves and branchlets of *Flindersia xanthoxyla* (*Rutaceae*), and the bark of *Lunasia amara* (*Rutaceae*).

REFERENCES.

Atkinson, N. and Rainsford, K. M. (1946): Austral. J. exp. Biol., 24, p. 49.
Atkinson, N. (1946): Nature, 158, p. 876.
Atkinson, N. (1949): Med. J. Austral., 1, p. 605.
Florey, H. W., Chain, E., Heatley, N. G., Jennings, M. A., Sanders, A. G., Abraham, E. P. and Florey, M. E. (1949): "Antibiotics," Vol. 1, Oxford University Press.
Webb, L. J. (1949): C.S.I.R.O. Bulletin No. 241.
Webb, L. J. (1952): C.S.I.R.O. Bulletin No. 268.

SALMONELLA BACTERIOPHAGES

3. TWO NEW BACTERIOPHAGES OF *S. ADELAIDE*

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(Accepted for publication 3rd October, 1955.)

In a proposed bacteriophage grouping scheme for strains of *S. adelaide*, Atkinson (1955) included two new *S. adelaide* phages Ad273 and Ad14, representing two new types of *S. adelaide* phage provisionally labelled type 2c and type 3. These two new phages were briefly described by Atkinson and Klauss (1954), who also found other phages resembling them. The present paper records some characteristics of the type 2c and type 3 phages.

METHODS.

Isolation of phages. The phages were obtained by growing suitable lysogenic strains of *S. adelaide* on agar with the *S. adelaide* indicator, strain 14 or Cey4, extracting the mixed growth in broth and centrifuging the extract which then contained the phage. Type 2c phages were obtained by growing *S. adelaide* strains of group 2c (Atkinson, 1955) with the indicator strain 14. Type 3 phages were obtained by growing *S. adelaide* strains of group 3 with the indicator strain Cey4.

Purification of phages. The centrifuged extracts, suitably diluted, were plated out on agar with strain Cey4 and well isolated plaques were picked off into broth with strain Cey4. The broths, after incubation, were again plated out with strain Cey4 and well isolated plaques again picked off into broth with strain Cey4. After incubation these broths were centrifuged, passed through a bacterial filter and stored in a refrigerator as stock phage preparations.

Investigations of phages. Lysis and lysogenicity tests were done by the methods of the grouping scheme (Atkinson, 1955). Heat resistance tests were done as described by Atkinson and Geytenbeek (1953) except that tests for lysis were made by spotting on strain Cey4. Citrate sensitivity tests were done according to Adams (1952). Antiserum neutralization tests were done by a method resembling that of Boyd (1950) and of Anderson and Felix (1953). Serial dilutions of the antiserum were mixed with a suitable dilution of phage producing semi-confluent lysis or many plaques in the control. The mixtures were placed at 48° C. for 1 hour and then spotted on the indicator strain Cey4. After incubation at 37° C. any lysis in the spots was noted. Complete neutralization was indicated by absence of lysis. Partial neutralization was indicated by reduction in the degree of lysis. The homologous phage was always included so that the comparative extent of neutralization of other phages could be seen.

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[1]This work was assisted by a grant from the National Health and Medical Research Council, Canberra.

Phage Ad273 was taken as typical of *S. adelaide* phage type 2c, and phage Ad14 as typical of *S. adelaide* phage type 3. The characteristics of these two phages were investigated extensively and other phages which appeared similar were compared with them.

Phage Ad273 was isolated from *S. adelaide* strain 273 (previously called S273 by Atkinson and Klauss, 1954) and phage Ad14 from *S. adelaide* strain 14. Purified stock phage preparations propagated on strain Cey4 were used in all tests.

RESULTS.

Lysis tests. The type strains of *S. adelaide* representing the six phage groups (Atkinson, 1955) were tested for lysis with phages Ad273 and Ad14. The strains tested were 2 (group 1a), 20 (group 1b), 37 (group 2a), 273 (group 2c), 14 (group 3) and Cey4 (group 4). The results of these tests are given in Table 1, which also includes the reactions of the *S. adelaide* phages of types 1a, 1b, 2a and 2b previously described.

TABLE 1.

Lysis tests with all known types of S. adelaide *phage.*

Phage tested	Strain tested					
	2 (group 1a)	20 (group 1b)	37 (group 2a)	273 (group 2c)	14 (group 3)	Cey4 (group 4)
New Phages						
Ad273 (type 2c)	+	+	+	−	+	+
Ad14 (type 3)	−	−	−	−	−	+
Previously described Phages						
Ad2 (type 1a)	−	+	+	+	+	+
Ad20 (type 1b)	+	−	+	+	+	+
Ad37H (type 2a)	+	+	−	−	+	+
Ad37C (type 2b)	+	+	−	−	+	+

+ = confluent or semi-confluent lysis.
− = no lysis.

Phage Ad273 (type 2c) lysed *S. adelaide* strains of all groups except group 2c, represented by strain 273 from which the phage was isolated. Phage Ad14 (type 3) lysed only strain Cey4 (group 4). Thus these two new types of phage differed widely in their host range and their lysis patterns were different from those of all the previously described phages included in Table 1.

Plaque characters. On strain 14 or Cey4, phage Ad273 formed very small plaques, average diameter 0·1 mm., with complete veil of secondary growth and a moderately regular edge. These plaques resembled those of the *S. adelaide* type 2a phage, Ad37H.

On strain Cey4, phage Ad14 formed larger and more obvious plaques than phage Ad273. The Ad14 plaques, of average diameter 0·5 mm., consisted of a clear, outer ring with irregular edge surrounding a central irregular spot of secondary growth.

Heat resistance. Preparations of phages Ad273 and Ad14 giving confluent lysis were heated for 30 minutes at 55° C. and 60° C. After heating at 55° C. activity was reduced, and after heating at 60° C. both phages failed to show any lysis. These phages were thus moderately heat sensitive and resembled the types 1b, 2a and 2b phages of Atkinson and Geytenbeek (1953).

Citrate sensitivity. Preparations of phage Ad273 producing confluent lysis in the control test without citrate failed to show any lysis in the presence of 0·05 M citrate. Phage Ad14 was unaffected by this concentration of citrate.

Serological tests. Phages Ad273 and Ad14 were tested for neutralization with antisera prepared against the phages Ad2, Ad20, Ad37H and Ad37C representing types 1a, 1b, 2a and 2b. Antisera were also prepared against the new phages Ad273 and Ad14 and used in neutralization tests against the other *S. adelaide* phages. The results of these tests are given in Table 2.

TABLE 2.

Antiserum neutralization tests with all known types of S. adelaide *phage.*

Phage tested	Antiserum tested					
	Ad2	Ad20	Ad37H	Ad37C	Ad273	Ad14
Ad273	−	−	−	+	+	−
Ad14	−	−	−	−	−	+
Ad2	+	+	−	+	tr	−
Ad20	+	+	−	−	tr	−
Ad37H	−	−	+	+	±	−
Ad37C	−	−	+	+	±	−

+ = neutralization to the same titre as the homologous phage.
± = neutralization at a lower dilution than the homologous phage.
− = no neutralization.
tr = some reduction in degree of lysis.

From the results in Table 2 no serological relationship was demonstrated between phage Ad14 and any other *S. adelaide* phage tested. Phage Ad273 was related to Ad37C and more remotely related to Ad37H.

Range of activity on other Salmonella *types and members of the* Enterobacteriaceae. The results of lysis tests with phages Ad273 and Ad14 on various members of the *Enterobacteriaceae* are given in Table 3.

Phage Ad14 showed a very restricted range of activity lysing only one strain each of *S. alachua, S. monschaui* and an Arizona paracolon type and two strains of *S. waycross. S. alachua* and *S. monschaui* have the O antigen 35 found in *S. adelaide,* and the Arizona strain has the O antigen 41 found in *S. waycross.* Occurrence of antigen 35 was not in itself sufficient, however, to render an organism sensitive to phage Ad14 as three strains of *E. coli* O 111, containing the Salmonella O antigen 35, were not lysed. Lysogenicity tests with these three strains on *S. adelaide* Cey4 failed to reveal any phage, so that their resistance was not due to carriage of phage like Ad14.

TABLE 3.

Lysis tests with phages Ad273 and Ad14 and members of the Enterobacteriaceae.

Organism tested	No. of strains	Phage used in test	
		Ad273	Ad14
S. paratyphi A	1	+	−
S. paratyphi B	2	+	−
S. typhi-murium	2	−	−
S. derby	2	−	−
S. muenchen	2	−	−
S. bovis-morbificans	3	+	−
	1	−	−
S. blegdam	2	−	−
S. enteritidis	1	−	−
	1	+	−
S. anatum	2	−	−
S. worthington	2	−	−
S. aberdeen	1	+	−
S. carrau	1	−	−
S. onderstepoort	1	−	−
S. newington	1	+	−
	1	−	−
S. senftenberg	1	−	−
S. potsdam	1	+	−
S. alachua	1	−	+
S. monschaui	1	+	+
S. waycross	2	+	+
	2	+	−
Ballerup (*S. ballerup*)	1	−	−
Bethesda Na11	1	−	−
Bethesda Md1	1	−	−
Arizona CDC 1715-50	1	−	+
Arizona 699-52	1	−	−
E. coli 0111	3	−	−
E. coli 055	1	−	−
E. freundii	1	−	−
Sh. boydii I	1	−	−
Sh. flexneri 1A	1	+	−
Sh. dispar	1	−	−
Pr. morgani	1	−	−
	1	−	−
S. adelaide 14		+	−
S. adelaide Cey4		+	+

+ = confluent or semi-confluent lysis or plaques.
− = no lysis.

Phage Ad273 lysed 10 different *Salmonella* types and *Shigella flexneri* 1A. It therefore possessed a considerable range of activity and thus resembled the type 2a phage Ad37H (Atkinson and Geytenbeek, 1953).

Occurrence of the new type 2c and type 3 phages. Type 2c phages like Ad273 were found only in the five American strains of *S. adelaide* which Atkinson and Klauss (1955) placed in group 2c. Strain 273, from which phage Ad273 came, was one of these American strains, and the other four were strains 274, 275, 276 and 277 (previously designated S274, S275, S276 and S277 by Atkinson

and Klauss, 1954). The phages from these four strains were considered to be identical with phage Ad273 on host range and plaque characteristics (Atkinson and Klauss, 1954).

Type 3 phages like Ad14 were found in many strains of *S. adelaide* by lysogenicity tests with strain Cey4. In all strains which Atkinson and Klauss (1955) placed in group 3, type 3 phage was detected and identified by host range and plaque characteristics. In strains of groups 1a, 1b, 2a and 2c, type 3 phage was also detected along with a second type of phage typical of the group (Atkinson and Klauss, 1954). In these strains the type 3 phages were obtained by picking off the type 3 plaques from the mixed phage plaques appearing in lysogenicity tests with strain Cey4. These purified preparations were then identified as before. In this way type 3 phages were found in 113 out of the 413 strains of *S. adelaide* grouped by Atkinson and Klauss (1955). The 113 strains consisted of 2 group 1a, 1 group 1b, 13 group 2a and 89 group 3 Australian strains, 5 group 2c American strains, one group 3 English strain and 2 group 3 Ceylon strains. In 279 Ceylon strains type 3 phage was not detected.

Thus type 2c phage was an uncommon type found only in five American strains whereas type 3 phage was a common type found in nearly all strains of *S. adelaide* from Australia, England and U.S.A., but seldom found in Ceylon strains.

Brief description of the new S. adelaide *type 2c and type 3 phages.*

Type 2c phage. The prototype selected was phage Ad273. This type of phage produced very small plaques completely covered in secondary growth resembling type 2a plaques (phage Ad37H). It showed some serological relationship to type 2a and 2b phages, it lysed strains of *S. adelaide* carrying type 2b phage (e.g., strain 37), but strains of *S. adelaide* carrying type 2c phage (e.g., strain 273) were resistant to lysis by type 2b phage. It rarely occurred naturally, and was found in strains of *S. adelaide* of group 2c together with type 3 phage. It was citrate and heat sensitive and lysed a number of other *Salmonella* types.

Type 3 phage. The prototype selected was phage Ad14. This type of phage produced small plaques with central secondary growth and clear outer zone. It showed no serological relationship to any other type of *S. adelaide* phage and lysed only strains of *S. adelaide* of group 4. All other groups were resistant to this phage as they all carried it. It occurred frequently and was found in natural lysogenic strains of groups 1a, 1b, 2a, 2c and 3 accompanied by other phages in groups 1a, 1b, 2a and 2c. It was citrate insensitive and heat sensitive. It had a restricted host range and lysed only one or two other members of the *Enterobacteriaceae.*

DISCUSSION.

The two new *S. adelaide* phages Ad273 and Ad14 differed from each other in all characteristics recorded here. Phage Ad273 showed, however, some

relationship to phages Ad37H and Ad37C (types 2a and 2b) in plaque morphology, serological and cross-resistance tests. Therefore it was placed as a subtype of type 2 (type 2c) rather than creating for it a new type. Phage Ad14, however, appeared different from all the other *S. adelaide* phages so far found and was therefore placed as a new type called type 3. Only four other representatives of type 2c phages were found, but numerous representatives of type 3 phage were detected. This phage was carried by most strains of *S. adelaide* except those from Ceylon. It was therefore difficult to detect unless susceptible Ceylon strains were available. However, on the indicator strain Cey4, it propagated exceedingly well and could easily be detected in lysogenic strains carrying it. Such lysogenic strains sometimes carried another phage also. This second phage was typical of the group to which the lysogenic strain was allotted (Atkinson, 1955).

Including the four types of *S. adelaide* phage described by Atkinson and Geytenbeek (1953), six types of *S. adelaide* phage have now been found and described. All came from natural lysogenic strains and they formed a family of heat sensitive phages divisible into 3 types (1, 2 and 3) of which types 1 and 2 were further divided into sub-types 1a and 1b, 2a, 2b and 2c.

SUMMARY.

Two new *S. adelaide* phages Ad273 and Ad14 were isolated from natural lysogenic strains of *S. adelaide*. Their host range, plaque characteristics, citrate sensitivity and serological reactions showed them to be different from each other and also from the *S. adelaide* phages of types 1a, 1b, 2a and 2b.

Phage Ad273 showed some relationship to the type 2a and 2b phages and was therefore allotted to a new sub-type of type 2 designated type 2c. Phage Ad14, which was serologically unrelated to type 1 or type 2 phages, was placed as a new type designated type 3.

Type 2c phages were found only in five American strains of *S. adelaide*. Type 3 phages were found in 113 strains of *S. adelaide* including Australian, English and American strains. They were rarely found in strains of *S. adelaide* from Ceylon.

REFERENCES.

Adams, M. H. (1952): J. Bact., 64, p. 387.
Anderson, E. S. and Felix, A. (1953): J. gen. Microbiol., 9, p. 65.
Atkinson, N. (1955): Austral. J. exp. Biol., 33, p. 371.
Atkinson, N. and Geytenbeek, H. (1953): *Ibid.*, 31, p. 441.
Atkinson, N. and Klauss, C. (1954): *Ibid.*, 32, p. 221.
Atkinson, N. and Klauss, C. (1955): *Ibid.*, 33, p. 375.
Boyd, J. S. K. (1950): J. Path. Bact., 62, p. 501.

PROTEIN METABOLISM AND HYPOGLYCAEMIA

by S. ROSE[1] AND J. NELSON

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(Accepted for publication 26th October, 1955.)

These experiments were designed to study the various mechanisms which may be involved in the increased rate of protein catabolism which occurs with hypoglycaemia. Insulin in small doses can promote protein anabolism (Engel, 1949) and recently Salter and Best have shown that insulin can cause growth and nitrogen retention in the hypophysectomized rat without the addition of any growth hormone (Salter and Best, 1953). However, Engel (1949) also showed that excess insulin caused an increased rate of protein catabolism which is probably due to the hypoglycaemia. This paper deals with the investigation of the possible mechanisms involved in this response.

METHODS.

Biological.

Engel *et al.* (1948) have developed and used (Engel, 1951b) a technique which can measure small and rapid changes in nitrogen metabolism of the rat. The method depends on the finding that under controlled circumstances the hourly rate of urea nitrogen accumulation in the bilaterally nephrectomized rat remains sufficiently constant for a long enough period to detect small changes in nitrogen metabolism. The validity and the results using the method are discussed in Engel's later paper (Engel, 1951b). The results could be expressed in terms of the rate of rise of blood urea or as milligrams of nitrogen per 100 gm. body weight per hour. Following Engel, the latter has been used. The method depends on the assumption that urea is the end product of protein catabolism and that it is equally distributed throughout the body water taken as 63 p.c. of the body weight (Moore, 1946; Pace and Rathbun, 1945). We have used this technique with certain modifications to suit the requirements of our own experiments.

Adult male rats, weighing between 200 and 250 gm., were fasted for 18 hours prior to being bilaterally nephrectomized. In some groups the operation was carried out under a short (10–15 minutes) intravenous pentothal anaesthetic. These animals were kept for 3 hours at 26° C. In the other groups the operation was carried out under nembutal anaesthesia and immediately after the nephrectomy they were given hourly repeated subcutaneous doses of nembutal (20 mg./100 gm./hour, representing about three times the maximum anaesthetic dose). These animals were deeply anaesthetized and required artificial respiration by intratracheal incubation connected to a suitable pump. We were able to arrange a method by which 8 animals could be maintained simultaneously. We found it necessary to suck mucus, etc., out of the trachea every hour. The animals were covered with cotton wool and placed in a chamber at 34–35° C. to maintain the body temperature at the normal 36° C.

[1]Working under a full-time grant from the National Health and Medical Research Council.

Hormone and glucose administration.

Adrenaline, hyperglycaemic factor and glucose were given as a continuous infusion into the jugular or portal vein. The infusion was made through a 26 G. dental needle attached to a polythene tube. This latter was connected to a syringe, the plunger of which was driven at a constant rate by an electric motor. We were able to provide simultaneous continuous infusion in 8 animals. This is of great value as experimental and control rats can be observed on the one day. All continuous infusions were given at the rate of 2 ml./hour. The control rats received 2 ml. of 0·9 p.c. sodium chloride per hour.

(1) Adrenaline hydrochloride (Parke Davis) was administered at a dose level of 3, 7 and 15 μg./100 gm./hour.

(2) Crystalline zinc insulin, free from hyperglycaemic factor, was given as a single intraperitoneal injection at a dose level of ½ unit/100 gm.

(3) Adrenocortical extract (Parke Davis Eschatin) 2 ml./100 gm. was given subcutaneously immediately and one hour after nephrectomy. Each ml. of this extract contains 200 μg. of cortisone.

(4) Crude hyperglycaemic factor was prepared according to the method of Sutherland and de Duve (1948). The potency was tested by intravenous injection into fed rabbits. The mean blood sugar rise in 6 rabbits 5 minutes after the injection of 1 mg. of the crude extract was 23 mg. p.c. The hyperglycaemic factor was given by continuous infusion into the portal vein at a dose level of 0·25 mg./hour.

(5) Glucose was given by continuous infusion at 30 and 120 mg./100 gm./hour.

Chemical analysis.

(1) Blood sugars were estimated on duplicate samples by the anthrone method (Prokhovnik and Nelson, 1953). The estimations were done on samples taken from the tail vein immediately and three hours after the nephrectomy. Portal vein blood was taken at the conclusion of the experiment by the introduction of a hypodermic needle high up in the portal vein (to ensure adequate mixing) and then 0·2 ml. of blood was slowly withdrawn. In the experiment, when glucose was being infused, the blood samples were taken whilst the infusion was still in progress.

(2) Blood urea was estimated on duplicate samples by the method of Archibald (1945).

(3) Muscle glycogen was estimated from a portion of the gastrocnemius muscle. Muscle and liver glycogens were estimated by the anthrone technique (Nelson and Prokhovnik, 1953).

RESULTS.

The effect of various treatments on the urea nitrogen accumulation, systemic and portal blood sugar and liver and muscle glycogen are shown in Table 1. Nineteen different groups of animals have been studied. The variables which separate the groups are:

(1) Experimental period after nephrectomy. This interval was usually the three hours immediately after the operation, but occasionally the interval between the eighteenth and twenty-fourth hour after operation was used. For descriptive purposes the two intervals will be designated "immediate" and "delayed."

(2) Intravenous pentothal was used for the short anaesthetic period. These animals were usually awake in 10–15 minutes. Nembutal at a dose level of 20 mg./100 gm./hour given hourly as subcutaneous injections was used to

produce the deep, prolonged anaesthesia. For descriptive purposes the two types of anaesthesia will be designated "short" or "deep and prolonged."

(3) Drug treatment. Insulin, adrenocortical extract (A.C.E.), adrenaline, hyperglycaemic factor and glucose were given in various ways. To facilitate the description of any one group, it will be numbered and in the Tables the variables are quoted after the group number.

Effect of large doses of insulin.

Half a unit of insulin/100 gm., given by intraperitoneal injection, produced a marked hypoglycaemic response, which was still present at the end of three hours, and an acceleration of protein catabolism (compare group 2 with 1). The same response occurs using the delayed experimental period (compare group 4 with 3). The basal level of urea nitrogen accumulation is the same immediately and 18 hours after nephrectomy (compare group 1 with 3). The accelerated rate consequent on insulin administration is slightly greater in the delayed period (compare group 2 with 4). These latter animals (group 4) had been starving and stressed for 18 hours longer and this may account for the increased response.

Effect of adrenocortical extract (A.C.E.).

Two ml. of A.C.E./100 gm. injected immediately and one hour after nephrectomy produced a very small increase in urea nitrogen production (compare group 5 with 1). This same response can be detected in those animals under deep, prolonged anaesthesia (compare group 6 with 15). Administration of A.C.E. did not potentiate the increased urea nitrogen retention produced by insulin hypoglycaemia in the immediate or the delayed experimental period (compare group 7 with 2 and 8 with 4).

Effects of adrenaline and hyperglycaemic factor.

Infusion of a small dose of adrenaline into the jugular or portal vein (3 μg./100 gm./hour) did not produce an acceleration of protein catabolism (compare groups 10 and 11 with 9 and 15). The infusion into the jugular vein produced a small increase in liver glycogen (compare group 10 with 9). Infusion of a larger dose of adrenaline (7 μg./100 gm./hour) into the portal vein, but not the jugular vein, resulted in a significantly higher rate of urea nitrogen production (compare group 12 with 1 and 13). Moreover, when the adrenaline was infused into the portal vein there was a decrease in the content of glycogen in the liver (compare group 13 with 12 and 9). A similar increase in urea nitrogen production was obtained with a still larger dose (15 μg./100 gm./hour) infused into the jugular vein (compare group 14 with 13). Infusion of hyperglycaemic factor into the portal vein had a similar effect to the intraportal infusion of adrenaline at the middle of the dose range. The rate of urea nitrogen production increased and the liver glycogen was depressed (compare group 16 with 9 and 13).

TABLE 1.

Group	No. of Animals	Experimental Time After Nephrectomy	Anaesthetic	Treatment	Results				
					mg./N_2/100 gm./hr.	Systemic Blood Glucose mg./100 ml.	Portal Blood Glucose mg./100 ml.	Liver Glycogen mg./100gm.	Muscle Glycogen mg./100gm.
1	10	immediate	short	nil	1·71±0·35*	—	—	—	—
2	8	immediate	short	insulin	3·56±0·56	106 initial 46 final	—	—	—
3	6	delayed	short	nil	1·88±0·52	—	—	—	—
4	8	delayed	short	insulin	4·29±0·72	—	—	—	—
5	6	immediate	short	eschatin	2·45±0·22	—	—	—	—
6	4	immediate	deep and prolonged	eschatin	2·60±0·33	—	—	—	—
7	6	immediate	short	insulin and eschatin	3·38±0·66	—	—	—	—
8	6	delayed	short	insulin and eschatin	3·40±0·66	—	—	—	—
9	4	immediate	deep and prolonged	intraportal saline	1·72±0·30	—	—	40	358
10	4	immediate	deep and prolonged	intravenous adrenalin 3 mg./100 gm./hr.	1·73±0·33	—	—	56	279
11	4	immediate	deep and prolonged	intraportal adrenalin 3 mg./100 gm./hr.	1·92±0·32	—	—	34	341
12	4	immediate	deep and prolonged	intravenous adrenalin 7 mg./100 gm./hr.	1·77±0·19	—	—	50	264
13	4	immediate	deep and prolonged	intraportal adrenalin 7 mg./100 gm./hr.	3·43±0·39	—	—	9	244
14	4	immediate	deep and prolonged	intravenous adrenalin 15 mg./100 gm./hr.	3·55±0·61	—	—	—	—
15	8	immediate	deep and prolonged	nil	1·74±0·41	—	—	—	—

TABLE 1—*continued.*

Group	No. of Animals	Experimental Time After Nephrectomy	Anaesthetic	Treatment	Results				
					mg./N_2/100 gm./hr.	Systemic Blood Glucose mg./100 ml.	Portal Blood Glucose mg./100 ml.	Liver Glycogen mg./100gm.	Muscle Glycogen mg./100gm.
16	4	immediate	deep and prolonged	intraportal hyperglycaemic factor	3·28±0·47	—	—	9	355
17	8	immediate	deep and prolonged	insulin	3·32±0·66	105 initial 45 final	—	—	—
18	6	immediate	deep and prolonged	insulin and intravenous glucose 120 mg./100 gm./hr.	1·69±0·22	107 initial 107 final	—	39	575
19	4	immediate	deep and prolonged	insulin and intraportal glucose 30 mg./100 gm./hr.	3·68±0·25	110 initial 60 final	99 final	8	348

• Standard Deviation.

Statistical Analysis:—

1. Nitrogen Accumulation.

For groups 1 & 2 $t = 8·54$ $P < 0·001$ For groups 4 & 8 $t = 2·25$ $P = 0·05$ For groups 13 & 14 $t = 0·35$ $P = 0·7$
3 & 4 $t = 6·6$ $P < 0·001$ 9 & 10 $t = 0·05$ $P > 0·8$ 9 & 16 $t = 4·35$ $P < 0·005$
1 & 3 $t = 2·15$ $P = 0·05$ 11 & 15 $t = 0·74$ $P = 0·5$ 13 & 16 $t = 0·44$ $P = 0·7$
2 & 4 $t = 2·26$ $P = 0·05$ 12 & 13 $t = 7·4$ $P < 0·001$ 1 & 15 $t = 0·14$ $P > 0·8$
1 & 5 $t = 4·5$ $P < 0·001$ 1 & 12 $t = 0·31$ $P = 0·7$ 15 & 17 $t = 5·42$ $P < 0·001$
6 & 15 $t = 3·84$ $P < 0·005$ 9 & 13 $t = 5·0$ $P = 0·005$ 1 & 18 $t = 0·05$ $P > 0·8$
2 & 7 $t = 0·5$ $P > 0·6$ 12 & 13 $t = 6·7$ $P < 0·001$ 9 & 19 $t = 7·67$ $P < 0·001$

2. Liver Glycogen.

For groups 9 & 10 $t = 0·66$ $P = 0·5$ For groups 9 & 16 $t = 5·05$ $P = 0·001$ For groups 13 & 16 $t = 0·21$ $P > 0·8$
9 & 18 $t = 0·25$ $P = 0·8$

3. Muscle glycogen.

For groups 9 & 18 $t = 4·54$ $P = 0·01$

Effect of prolonged, deep anaesthesia.

The basal level of urea production remains the same (compare group 1 with 15) and the increased rate consequent on insulin administration still occurs (compare group 15 with 17). In those animals with deep and prolonged anaesthesia there is a greater individual variation in any one group. Part of the explanation for this may lie in the difficulty of maintaining an even body temperature throughout the experimental period.

Effect of glucose administration.

Glucose given as a continuous intravenous infusion at 120 mg./100 gm./hour prevents the fall in blood sugar produced by insulin and the rate of urea nitrogen production does not increase over the basal figure (compare group 18 with 15 and 1). In those rats receiving insulin, the muscle, but not the liver glycogen content is higher with the glucose administration than with a corresponding control infusion of saline (compare group 18 with 9). Glucose given as a continuous intraportal infusion at the rate of 30 mg./100 gm./hour did not prevent the systemic fall in blood sugar following insulin, but the portal blood was maintained at normoglycaemic levels (group 19). In the latter case the urea nitrogen production was accelerated above basal levels (compare group 19 with 9). Moreover, despite the normoglycaemic level in the portal vein, the liver glycogen content was lower than the control (compare group 19 with 9) and the muscle glycogen had not altered significantly.

DISCUSSION.

In the intact animal the increased protein catabolic response may be due to several mechanisms taking part together. Three types of experiments are available to study this situation:

(1) Observation of the intact animal to determine if the particular process does occur during insulin hypoglycaemia. A negative result is decisive, but a positive finding may not establish a direct causal relationship between the mechanism and the increased protein catabolic response.

(2) Experimental interference with one or more mechanisms during insulin hypoglycaemia. The interpretation of such a result must take into account the possible permissive action of the mechanism. That is, the mechanism may not be the exciting or regulatory agent but in its absence the overt signs of the response fail to occur.

(3) Experimental activation of a mechanism or its stimulation by drug administration in an animal not developing insulin hypoglycaemia. If these results are to have physiological rather than pharmacological significance, due attention must be given to the dose, site and mode of administration of the drug. We have tried to keep these factors in mind in the design and interpretation of our experiments and the appraisal of other papers in this field.

Rôle of the adrenal cortex.

Ingle has proposed the important concept that the adrenal cortex is necessary for but not responsible for the increased nitrogen excretion after stress (Ingle, 1949). The permissive action of the adrenal hormones has since been shown to be a more general phenomenon. Engel (1951b) has developed this concept to include the relationship between the adrenal cortex and the increased protein catabolism which accompanies insulin hypoglycaemia. Part of the evidence for this concept is the finding that stress and insulin hypoglycaemia, but not A.C.E. or A.C.T.H., induce an immediate (measurable within 3 hours) change in nitrogen metabolism. Our own results confirm this finding. A.C.E. and A.C.T.H. induce a delayed increase in protein catabolism which can only be detected in the second three-hour period (Engel, 1951b). When A.C.E. was given with insulin, Engel observed a more rapid and increased protein catabolic response than with either drug given alone (Engel, 1949). We have not been able to show this potentiating effect of A.C.E. administration. Our rats receiving 0·5 unit of insulin/100 gm. by intraperitoneal injection showed a greater and more rapid increase in urea nitrogen production than Engel's animals similarly treated with subcutaneous insulin (Engel, 1949). Engel (1951a) has shown that a small stress is potentiated by the presence of excess adrenal hormones, but the acceleration of nitrogen metabolism by a large stress is unaltered by adrenal hormone over-dosage. Since our animals receiving intraperitoneal insulin responded as though to a large stress, this may explain why A.C.E. did not have a potentiating effect.

Engel and others have also studied the level at which the adrenal cortex acts on nitrogen metabolism. The conclusion is reached that the adrenal cortex acts predominantly, but not only, extrahepatically at the level of whole protein so that amino acids are mobilized from the peripheral tissues (Engel, 1951b and 1953).

Rôle of adrenaline.

Many workers have now shown that the level of adrenaline in the blood is higher in the hypoglycaemic than the normal animal (Weil Malherbe, 1953). A hundred microgrammes of adrenaline injected subcutaneously into a rat produced an immediate increase in urea formation (Engel, 1948). We have shown that a similar response occurs in the anaesthetized rat given 15 μg./100 gm./hour as a continuous infusion into the jugular vein. Adrenaline can stimulate the pituitary-adrenal axis but Engel has shown that the protein catabolic response is not directly related to the increased secretion of adrenal cortical hormone (Engel, 1951). Thus he has shown that stress and adrenaline induce an immediate change in nitrogen metabolism, A.C.T.H. and A.C.E. a delayed response. Moreover, A.C.E. and A.C.T.H. administration are associated with a rise in plasma amino nitrogen (Li *et al.*, 1949), whilst stress (Farr *et al.*,

1942; Mann *et al.*, 1946) and adrenaline (Friedberg and Greenberg, 1948) cause a fall in plasma amino-nitrogen. This seems to indicate that adrenaline and stress act to increase the rate of removal (probably by the liver) of amino acids from the blood. This is of great interest in view of our results which show that a particular dose of adrenaline (7 μg./100 gm./hour) can increase the rate of protein catabolism when injected intraportally but not when given via the jugular vein. Stress can induce an increased protein catabolism in the adrenalectomized rat maintained on cortisone (Ingle, 1949). The response in this case obviously cannot be due to the release of adrenaline by the adrenal medulla. In view of the above results, particularly with intraportal infusion of adrenaline, one might postulate a local release of adrenaline from the sympathetic nerves to the liver under stressed conditions. The finding that various neural lesions result in liver glycogenolysis and hyperglycaemia (Bernard, 1849; Ogilvie, 1952) could support such a thesis.

The question naturally arises as to the relation between the protein catabolic and the liver glycogenolytic effect of adrenaline. Sherlock (1949) has shown that 2 μg. of adrenaline/100 gm./hour infused into the femoral or portal vein do not cause a decrease in liver glycogen content. We have confirmed this finding using 3 μg./100 gm./hour and with this dose there is no increase in the rate of protein metabolism. However, when the adrenaline dose is increased to 7 μg./100 gm./hour, given intraportally, the liver glycogen content decreases and there is also a rise in the rate of protein catabolism. This response does not occur with the same dose given via the jugular vein. If the adrenaline dose is increased still further (15 μg./100 gm./hour) the same response can be obtained with systemic administration of the drug. These results suggest a relationship between the liver glycogen content and the rate of protein catabolism. However, the significance of these findings might be questioned because of the very low glycogen content of our deeply anaesthetized control rats.

Deep nembutal anaesthesia (approximately three times the anaesthetic dose) did not prevent the increased rate of urea nitrogen production which normally occurs with insulin hypoglycaemia. At this dose level adrenaline release and sympathetic activity should be prevented (Banerji and Reid, 1933; Keeser and Keeser, 1935; Trethewie and Gladwell, 1952). These results indicate that even though adrenaline discharge may play a part in the intact animal, such a mechanism is not indispensable for the protein catabolic response of insulin hypoglycaemia.

Rôle of hyperglycaemic factor.

The hyperglycaemic factor which can be extracted from the pancreas, and to a lesser extent the gastric and intestinal mucosa, is glycaemic and acts directly on the liver (Pincus, 1950). Most of the data indicate that this factor acts on the liver in a similar fashion to adrenaline by increasing the activity of the phosphorylase system (Sutherland and Cori, 1948). This factor may be released

into the portal circulation during insulin hypoglycaemia (Foà *et al.*, 1952). When the crude pancreatic hyperglycaemic extract was infused into the portal vein the glycogen content of the liver was decreased and the protein catabolic rate was increased. This supports the view given above that there may be a relationship between the liver glycogen content and protein catabolism.

Rôle of hypoglycaemia.

The above results have indicated a possible relation between carbohydrate availability to the liver and protein metabolism. We therefore perfused glucose (30 mg./100 gm./hour) into the portal vein of rats who had received a large dose of insulin. The systemic hypoglycaemia was not prevented but the portal blood was maintained at normoglycaemic level. Despite this the liver glycogen content was lower than normal and the urea nitrogen production was accelerated. This occurred in the deeply anaesthetized rats where adrenaline release was probably completely inhibited. A possible explanation may be that the hyperglycaemic factor was released from the pancreas which was receiving hypoglycaemic blood. In the presence of this factor the liver glycogen falls despite normoglycaemic blood reaching it by the portal vein.

SUMMARY.

Insulin hypoglycaemia results in an increased rate of urea nitrogen accumulation in the nephrectomized rat. The possible mechanisms involved in this response have been studied.

(1) The protein catabolic response is not caused directly by the increased adrenocortical activity which accompanies hypoglycaemia.

(2) Infusion of adrenaline increases the rate of urea nitrogen accumulation. The main action of adrenaline in this regard seems to be in the liver. 7 μg./100 gm./hour infused into the portal, but not the jugular, vein elevates the rate of protein catabolism and depresses the liver glycogen content. The protein catabolic response occurs despite deep nembutal anaesthesia. This indicates that adrenaline discharge is not the only mechanism involved.

(3) A crude pancreatic hyperglycaemic extract perfused into the portal circulation elevates the level of protein catabolism. The results from (2) and (3) indicate a relation between the glycogen content of the liver and protein catabolism.

(4) Maintaining normoglycaemic portal blood levels in the face of systemic hypoglycaemia does not prevent the increased protein catabolism and depressed liver glycogen in the deeply anaesthetized rat.

Acknowledgments. We are grateful to Mr. S. Prokhovnik for his technical assistance and to Nicholas Pty. Ltd. for making his time available to us. The crystalline insulin was obtained from the Commonwealth Serum Laboratories through the courtesy of Dr. Porter.

REFERENCES.

Archibald, R. M. (1945): J. biol. Chem., 157, p. 507.
Banerji, H. and Reid, C. (1933): J. Physiol., 78, p. 370.
Bernard, C. (1849): Comp. rend. Soc. Biol., 1, p. 14.
Engel, F. L. *et al.* (1948): J. biol. Chem., 174, p. 99.
Engel, F. L. (1949): Endocrinology, 45, p. 170.
Engel, F. L. (1951a): *Ibid.*, 49, p. 538.
Engel, F. L. (1951b): Recent Progress in Hormone Research, 6, p. 277.
Engel, F. L. (1953): Physiol. Revs., 15, p. 397.
Farr, *et al.* (1942): Proc. Soc. exp. Biol., 50, p. 256.
Foà, P. P. *et al.* (1952): Amer. J. Physiol., 171, p. 32.
Friedberg, F. and Greenberg, D. M. (1948): J. biol. Chem., 168, p. 405.
Ingle, D. J. (1949): Annals N.Y. Acad. Science, 50, p. 576.
Keeser, E. and Keeser, I. (1935): Archiv. exp. Path. Pharmakol., 179, p. 226.
Li, C. H. *et al.* (1949): J. biol. Chem., 177, p. 91.
Mann, E. *et al.* (1946): J. clin. Invest., 25, p. 701.
Moore, F. D. (1946): Science, 104, p. 157.
Ogilvie, R. F. (1952): Vitamins and Hormones, 10, p. 183.
Pace, N. and Rathbun, E. N. (1945): J. biol. Chem., 158, p. 685.
Pincus, I. J. (1950): J. clin. Endocrinol., 10, p. 556.
Prokhovnik, S. and Nelson, J. (1953): Austral. J. exp. Biol., 31, p. 279.
Salter, J. and Best, C. H. (1953): Brit. med. J., 2, p. 353.
Sherlock, Sheila (1949): Amer. J. Physiol., 157, p. 52.
Sutherland, E. W. and de Duve, C. (1948): J. biol. Chem., 175, p. 663.
Sutherland, E. W. and Cori, C. F. (1948): *Ibid.*, 172, p. 737.
Trethewie, E. R. and Gladwell, P. G. (1952): Austral. J. exp. Biol., 30, p. 52.
Weil Malherbe, H. (1953): J. Physiol., 122, p. 428.

THE PANCREATIC LESION IN ADULT MICE INFECTED WITH A STRAIN OF PLEURODYNIA VIRUS

4. FURTHER STUDIES UPON THE RESPONSE OF THE PANCREAS TO THE SIMULTANEOUS PRODUCTION OF AMYLASE AND VIRUS

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(Accepted for publication 7th November, 1955.)

Previous studies (Dineen and Robertson, 1955) showed that the pancreatic amylase level was higher, and the rate of amylase regeneration greater, during the 36 hours following the intraperitoneal injection of the PL.5 virus into adult mice. Dineen and Barter (1953) and Robertson (1954) showed that the changes in the pancreatic acinar cells reached their greatest complexity at 36 to 48 hours following the injection of the virus.

The response of the adult mouse pancreas to the stress of producing both amylase and PL.5 virus during the phase of maximum complexity in the histological evolution of the disease process is recorded in the following studies.

MATERIALS AND METHODS.

The preparation of the infant mouse muscle suspensions of the PL.5 virus and the method for estimating amylase in the pancreas have been described in the earlier communication (Dineen and Robertson, 1955).

EXPERIMENTAL.

Amylase levels were determined in pancreases removed from non-infected animals and from animals infected with the PL.5 virus for 48 and 72 hours, both before and one and four hours after treatment with pilocarpine.

Eighteen 10-week old male mice were randomly distributed in two groups of nine animals. One group of animals was injected intraperitoneally with 0·2 ml. of a 20 p.c. infant mouse muscle suspension of the PL.5 virus 48 hours before estimation of the pancreatic amylase levels. The other group was not injected with virus.

Three animals from each of the two groups were set aside and the remaining 12 animals were injected five times with 0·1 ml. of an 0·1 p.c. pilocarpine nitrate solution in distilled water at consecutive 15-minute intervals.

[1]This work was carried out with support from the Medical Research Committee of the University of Adelaide and by the John Mortlock Medical Bequest.
[2]In receipt of a grant from the National Health and Medical Research Council, Australia.

TABLE 1.

Pancreatic amylase titres of infected and non-infected animals before and one and four hours after treatment with pilocarpine.

Hours after virus injection	Hours after pilocarpine treatment	Replications						
		1	2	3	4	5	6	Ave.
Non-infected	Not treated	3·269*	3·392	3·498	3·576	3·306	3·625	3·444
48 hours		3·371	3·229	3·049	3·248	3·246	3·283	3·071
72 hours		2·862	2·356	2·977	2·594	3·107	2·854	2·792
Non-infected	1 hour	3·059	3·239	3·328	3·168	3·153	3·190	3·189
48 hours		3·204	2·728	3·112	2·815	2·618	3·283	2·960
72 hours		3·061	2·111	2·501	2·726	<1·778†	2·609	<2·602
Non-infected	4 hours	3·326	3·178	3·402	3·002	3·148	3·364	3·237
48 hours		3·220	2·688	2·490	3·029	3·283	3·283	2·999
72 hours		2·769	<1·778	<1·778	2·312	<1·778	<1·778	<2·541

* The amylase titres are recorded as the $\log_{10}$ of the reciprocal of the end-point dilution.

† Although amylase activity was detectable, the end-point was not included in the dilution range.

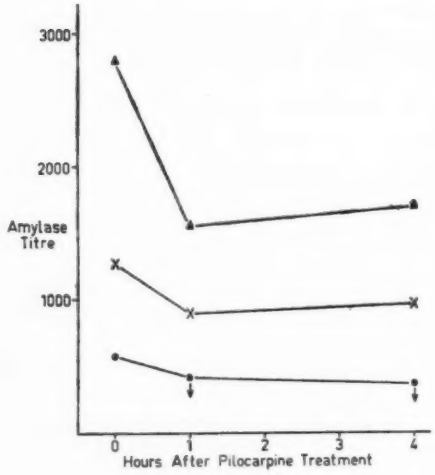

Fig. 1. Average amylase levels in pancreases removed from infected and non-infected animals before and after pilocarpine treatment. The amylase titre is expressed as the reciprocal of the end-point dilution.

▲ Non-infected.
× Infected for 48 hours.
● Infected for 72 hours.
⁝ Amylase titre less than the figure recorded.

Immediately after the administration of the pilocarpine, the six animals not treated were exsanguinated by decapitation and the pancreases removed and pooled in their appropriate groups for estimation of the amylase content. Three animals were selected at random from the two groups and at one and four hours after the administration of the pilocarpine, and the pancreases were pooled, weighed and the amylase titres determined. This experiment was repeated six times.

Similarly, estimations were carried out on pooled pancreases which were removed from animals infected for 72 hours, both before and one and four hours after pilocarpine treatment.

During the course of this work the serial infant mouse passage of the virus progressed from the 36th to the 38th passage.

The amylase titres and their graphical representation are given in Table 1 and Fig. 1.

The results given in Table 1 and represented in Fig. 1 show that the pancreatic amylase levels both before and after pilocarpine treatment were higher in the non-infected animals than in the animals infected for 48 and 72 hours. The results also suggest that regeneration of amylase has not occurred in the non-infected and the infected animals during the four hours following pilocarpine treatment.

DISCUSSION.

The analysis of the amylase titres in the pancreases removed from the infected and the non-infected animals, before and one and four hours after the administration of pilocarpine, is given in Table 2.

TABLE 2.

Analysis of variance of the pancreatic amylase titres of the non-infected animals and the animals infected for 48 hours, before and one and four hours after pilocarpine treatment.

Source of variation	Degrees of freedom	Sum of squares S.S.	Mean square M.S.
Between pilocarpined	1	6,709	6,709
Non-pilocarpined vs. pilocarpined	1	212,827	212,827 ($0{\cdot}05<P<0{\cdot}10$)
Pilocarpine treatment (B)	2	219,536	
Non-infected vs. infected (A)	1	706,702	706,720 ($P<0{\cdot}01$)
A × B	2	39,119	19,560
Treatments	5	965,375	193,075 ($0{\cdot}01<P<0{\cdot}05$)
Replications	5	90,384	
Replications × treatments	25	1,917,431	
Error	30	2,007,815	66,927
Total	35	2,973,190	

2·000 was subtracted from the log. titres and the differences multiplied by 1,000.

The M.S. for the comparison of the amylase titres in the non-infected and the infected pancreases was significant at the 0·1 p.c. level. Thus the level of amylase was higher in the pancreases removed from the non-infected animals than in the pancreases removed from the animals infected for 48 hours.

The results represented in Fig. 1 suggested that a more vigorous response to the effect of the injection of pilocarpine occurred in the non-infected than in the infected pancreases. However, the M.S. (19,560) calculated for the interaction of the comparison non-infected vs. infected (A), and pilocarpine treatment (B), did not conflict with the hypothesis that the same response occurred in the pancreases of the infected and the non-infected animals following pilocarpine treatment.

In order that the results of the present studies might be correlated with the responses which were recorded in the pancreases removed from animals infected for 12, 24 and 36 hours, it was necessary to demonstrate that no change in technique had occurred. Consequently, the response of the pancreases of the non-infected group of animals of the present series to the effect of pilocarpine administration was compared with the results obtained during the earlier experiments. The appropriate analysis is given in Table 3.

TABLE 3.

Analysis of variance of the pancreatic amylase titres of the non-infected groups of animals of the present series (A) and the earlier experiments (B) and before and one and four hours after pilocarpine treatment.

Source of variation	Degrees of freedom	Sum of squares S.S.	Mean square M.S.
Technique A vs. B	1	14,455	
Pilocarpine treatment	2	350,828	175,414 ($P<0\cdot01$)
Technique × pilocarpine treatment	2	7,291	
Treatments	5	372,574	74,515 ($0\cdot01<P<0\cdot05$)
Replications	3	44,365	
Treatments × replications	15	281,029	
Error	18	325,394	18,077
Total	23	697,968	

3·000 was subtracted from the log. titres and the differences multiplied by 1,000.

Table 3 shows that the significant M.S. (193,075) for treatments was due to the effect of the injection of pilocarpine and that no detectable change of the amylase levels or the response of the pancreases to pilocarpine treatment had occurred in the non-infected animals during the experiments. The analysis also shows that the method of the amylase titration was reproducible. Consequently, the results of the present studies were correlated with the earlier work reported by Dineen and Robertson (1955).

Dineen and Robertson showed that (1) the titre of pancreatic amylase was *higher* in the animals infected for 24 and 36 hours than in the non-infected animals; (2) pilocarpine treatment reduced the level in both infected and non-infected animals to the same level; and (3) enzyme regeneration was faster in the animals infected for 12, 24 and 36 hours than in the non-infected animals and regeneration occurred in the infected pancreases during the first four hours following pilocarpine treatment.

The present studies showed that (1) the titre of pancreatic amylase was *lower* in the animals infected for 48 and 72 hours than in the non-infected animals; (2) no detectable difference occurred between the response of the pancreatic amylase levels of the non-infected animals, and the animals infected for

48 hours; and (3) no significant regeneration occurred in the pancreases removed from the non-infected animals or the 48 hours infected group during the four hours following pilocarpine treatment. (The M.S. (6,709) which was appropriate to test a difference of the amylase levels at one and four hours after pilocarpine treatment, was not significant.)

During the morphological studies (Dineen and Barter, 1953), structurally normal acini were recognized in the infected pancreases. These acini were more readily observed during the later stages of the infection when the inflammatory response had subsided. However, it is likely that the intact acini were present throughout the course of the virus infection, and that the low titre, normal response of the 48 hours infected pancreases, following the administration of pilocarpine, was related to the action of the intact acini. These structurally normal acini appear, therefore, to be functionally resistant to infection with the PL.5 virus.

SUMMARY.

The present studies showed that (1) the titre of pancreatic amylase was lower in the animals infected for 48 and 72 hours than in the non-infected animals; (2) no detectable difference to pilocarpine treatment occurred between the response of the pancreatic amylase levels of the non-infected animals and the animals infected for 48 hours; and (3) no significant regeneration occurred in the pancreases removed from the non-infected or the 48 hours infected group during the four hours following pilocarpine treatment.

The low titre, but normal response of the 48 hours infected pancreases, following the administration of pilocarpine, was probably related to the presence of some intact acini.

Acknowledgment. The writers are indebted to Mr. A. Boyd for the care of the animals.

REFERENCES.

Dineen, J. K. and Barter, R. A. (1953): Austral. J. exp. Biol., 31, p. 337.
Dineen, J. K. and Robertson, J. S. (1955): *Ibid.*, 33, p. 567.
Robertson, J. S. (1954): *Ibid.*, 32, p. 393.

THE PRODUCTION OF PROLONGED HYPOCALCAEMIA IN THE RABBIT

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(Accepted for publication 26th October, 1955.)

In a previous communication Kerr Grant (1953) described three patients with hypoparathyroidism in whom fits, raised intracranial pressure and papilloedema were prominent features. He showed that all these manifestations could be cured by adequate control of the hypocalcaemia and suggested a mechanism which may explain them. However, their exact physico-chemical pathogenesis remained unknown. These studies, performed in the Clinical Research Unit of the Royal Prince Alfred Hospital, Sydney, have now been extended, using the rabbit as an experimental animal.

The production of prolonged hypocalcaemia by the operation of parathyroidectomy is difficult in the rabbit and this is largely due to the variable position of the inferior ("external") parathyroid glands (Engfeldt, 1950). Dietary restriction of calcium alone is inadequate. Hence it was decided to attempt to produce hypocalcaemia by repeated injections of disodium ethylenediamine tetraacetic acid (disodium EDTA).

Ethylenediamine tetraacetic acid (EDTA) forms soluble non-ionized complexes with many cations and behaves as a chelating agent (Schwarzenbach and Ackermann, 1948; Wade and Burnum, 1955). Popovici, Geschickter, Reinovsky and Rubin (1950) have repeatedly shown by experiments *in vitro* that at a physiological pH in human or rabbit serum EDTA combines with calcium ion stoichiometrically and that the calcium so bound to EDTA is no longer precipitated by the addition of oxalates. The same workers have also demonstrated that administration of the neutral sodium salt of EDTA to the rabbit *in vivo* causes a fall in the serum calcium level, and, if given in sufficient dosage, may cause hypocalcaemic tetany and death. The rate of fall of the serum calcium, and its duration, depend on the route of administration as well as the dose and the rapidity with which it is given; it is most rapid following intravenous injection and slowest and most prolonged when the salt is given subcutaneously. The calcium disodium EDTA formed is readily excreted by the kidney (Foreman, Vier and Magee, 1953; Spencer, Vankinscott, Lewin and Laszlo, 1952).

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[1]National Health and Medical Research Council Research Fellow.

[2]Supported in part by the National Health and Medical Research Council of Australia.

Methods and Results.

The experimental animals were sexually mature male and female rabbits bred at the animal farm of the hospital. They were fed throughout on a standard mixed diet supplied as dry pellets and given tap-water in unrestricted quantities.

The solution for injection was prepared as follows: 20 gm. of chemically pure disodium EDTA were dissolved in 100 ml. of hot water and stored in a rubber-stoppered bottle. Although the solubility of disodium EDTA in cold water is only about 11 gm. p.c., it was found convenient to use a 20 p.c. solution to avoid excessive volume of individual injections. Fortunately the salt remains in solution after cooling to body temperature long enough to allow injections of the supersaturated solution to be given. The stock solution must, of course, be heated before each injection.

Blood was obtained by ear vein puncture and the serum calcium was determined by the method of Fales (1953).

In preliminary experiments injections of 200–400 mg./kg. were given subcutaneously once daily for 3–4 days. Each injection was followed by a pronounced fall of serum calcium which reached its lowest point 7–8 hours after injection, but often did not return to the pre-injection level after 24 hours. Some animals developed severe hypocalcaemia with tetany and died.

Thus it was decided to give daily subcutaneous injections of disodium EDTA in a dose of 150–200 mg./kg. Blood for analysis was taken immediately before these injections; it will be realised that for part of each day the serum calcium level was lower than that of the specimen collected.

At the time of writing significant hypocalcaemia of 4–8 weeks' duration has been induced in 6 rabbits by this technique. Further groups of animals are now being similarly treated.

Fig. 1 is a graphic representation of a typical experiment which was electively terminated. Once hypocalcaemia was established it was maintained at such a level that the serum calcium was always 3–4 mg. p.c. lower than before treatment.

The Figure also illustrates two other important points of technique. Firstly, it was soon discovered that it is desirable to use intermittent courses of injections (with rest days)

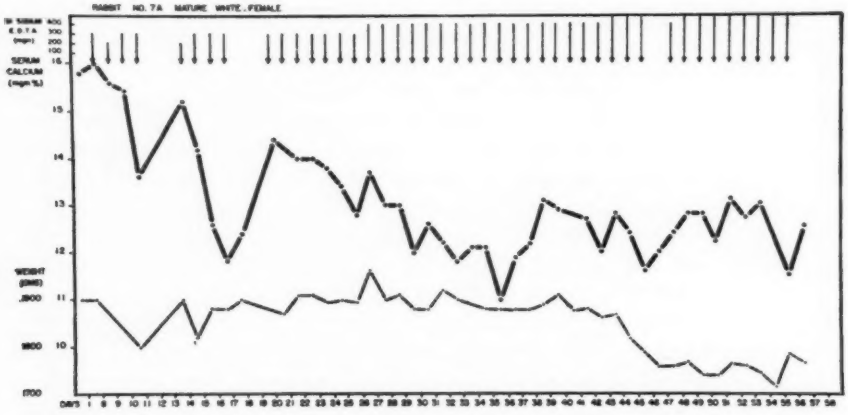

Fig. 1. The response of serum calcium and body weight to repeated subcutaneous injections of disodium ethylenediamine tetraacetic acid.

during the first two weeks of the treatment. In some of the earlier experiments the animals refused to eat or drink after continuous daily injections and died of acute dehydration and inanition. Secondly, it was found to be necessary to increase the dosage of disodium EDTA in order to achieve a comparable effect as the weeks passed. This may be due to parathyroid hypertrophy in response to hypocalcaemia, calcium being thus more readily mobilised from the bones.

DISCUSSION.

This technique has a number of obvious advantages. It is effective and relatively simple. The solution does not require to be sterilized and the syringe and needles are simply boiled each day before use. There has never been any sepsis following injections. In addition, when it is desired to terminate hypocalcaemia, it is only necessary to cease the injections. Parathyroid extract or vitamin D with calcium need not be given.

Some possible disadvantages require consideration. The aqueous solution of disodium EDTA has a pH of about 5 and produces a moderately severe local reaction, sometimes with formation of haematomas, when given subcutaneously. For some experiments the pH of the solution for injection was adjusted to 7·4 by the addition of 10 N. NaOH. At this pH 20 gm. of sodium EDTA are soluble in 100 ml. of cold water but the solution produced a similar local reaction and the systemic hypocalcaemic action did not seem to be as prolonged. This solution is no longer used.

Of more importance is the possibility that EDTA or its salts may have direct toxic actions on the animal in the doses given. The acute toxicity of disodium EDTA is due to its action in producing hypocalcaemia. The acute and subacute toxicity of calcium disodium EDTA is quite low in a number of different species. Bauer, Rullo, Spooner and Woodman (1952) showed that no rabbit died when calcium disodium EDTA was given intravenously in a dose of 2 gm./kg. and that the dose required to kill 50 p.c. of a test group, when given by the intraperitoneal route, exceeded 6 gm./kg. Further, when groups of rabbits were given daily intraperitoneal injections of 0·5 gm./kg. of calcium disodium EDTA, 50 p.c. died in an average time of 78 days, but there was no significant mortality before the 59th day. The duration of these experiments has not exceeded 56 days in any case, and in none has the dose of disodium EDTA exceeded 400 mg./kg. given subcutaneously.

Finally, EDTA will combine with other cations besides calcium so that the specificity of the response might be questioned. Of these other cations magnesium is probably the most important in the interpretation of the changes which occur in the animal in prolonged hypocalcaemia. No determinations of serum magnesium have yet been performed on these rabbits; but it has been shown that at a physiological pH disodium EDTA combines poorly with magnesium in the presence of calcium (Schwarzenbach and Ackermann, 1948; Popovici, Geschickter, Reinovsky and Rubin, 1950; Wade and Burnum, 1955).

Furthermore, when magnesium disodium EDTA is given to the rabbit in sufficient dosage, the magnesium in the complex is actually displaced by circulating ionized calcium so that the serum calcium level falls appreciably while the serum magnesium level rises above normal (Popovici, Geschickter and Rubin, 1951—seen in abstract only).

SUMMARY.

A technique is described for the production of prolonged hypocalcaemia in the rabbit by the daily subcutaneous injection of a 20 p.c. solution of disodium EDTA. The advantages and disadvantages of the method are briefly discussed.

Acknowledgments. I should like to express my thanks to Dr. C. R. B. Blackburn, Director of the Clinical Research Unit, Royal Prince Alfred Hospital, Sydney, for his constant advice and criticism of the research programme of which this technique is a part. Thanks are also extended to Miss P. Crowe, B.Sc., for necessary assistance with the animals, and to Miss B. James, Secretary.

REFERENCES.

Bauer, R. O., Rullo, F. R., Spooner, C. and Woodman, E. (1952): Fed. Proc., 11, p. 321.
Engfeldt, B. (1950): Acta Endocrinol., Supp. 6.
Fales, F. W. (1953): J. biol. Chem., 204, p. 577.
Foreman, H., Vier, M. and Magee, M. (1953): *Ibid.*, 203, p. 1045.
Grant, D. K. (1953): Quart. J. Med., n.s. 22, p. 243.
Popovici, A., Geschickter, C. F., Reinovsky, A. and Rubin, M. (1950): Proc. Soc. exp. Biol. Med., 74, p. 415.
Popovici, A., Geschickter, C. F. and Rubin, M. (1951): Bull. Georgetown Univ. Med. Centre, 5, p. 108.
Schwarzenbach, G. and Ackermann, H. (1948): Helv. chim. Acta, 31, p. 1029.
Spencer, H., Vankinscott, V., Lewin, I. and Laszlo, D. (1952): J. clin. Invest., 31, p. 1023.
Wade, J. F., Jnr. and Burnum, J. F. (1955): Ann. int. Med., 42, p. 251.

THE PRESSURE AND CALCIUM CONCENTRATION OF THE CEREBROSPINAL FLUID OF THE RABBIT

by L. C. A. WATSON[1] AND C. R. B. BLACKBURN

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(Accepted for publication 26th October, 1955.)

Involvement of the central nervous system is not uncommon in hypoparathyroidism and humans may develop epileptic fits, mental deterioration, coma, electroencephalographic abnormalities, raised intracranial tension, papilloedema and intracranial calcification. All these abnormalities have been studied in this Unit and the clinical investigation of three patients in whom fits were associated with papilloedema was described by Kerr Grant (1953); however, the exact physico-chemical mechanism or mechanisms responsible for such varied changes remain largely unknown.

As there are obvious advantages in using experimental animals for the study of this problem, a technique was developed for the production of prolonged hypocalcaemia in the rabbit (Watson, 1956). In this way, fits and raised intracranial tension can be produced in the rabbit (Watson — unpublished observations).

The present communication contains (a) the description of a technique for the registration of the cerebrospinal fluid pressure and the collection of uncontaminated fluid from the rabbit, and (b) some observations on the pressure and calcium concentration of the cerebrospinal fluid in a group of normal rabbits. The changes which occur in hypocalcaemia will be the subject of a future paper.

MATERIALS AND METHODS.

The experimental animals were sexually mature male and female rabbits bred at the animal farm of the hospital. They were fed on a standard mixed diet supplied as dry pellets and given tap-water in unrestricted quantities.

The calcium concentration of serum and cerebrospinal fluid was determined by the micro-method of Fales (1953).

Cisternal puncture was performed under nembutal anaesthesia. Short flat-bevelled needles were used to enter the cisternal space. Two sizes (gauges 20 and 25) have been tried, but in the later experiments a 25 gauge needle was used exclusively.

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[1]National Health and Medical Research Council Research Fellow.

[2]Supported in part by the National Health and Medical Research Council of Australia.

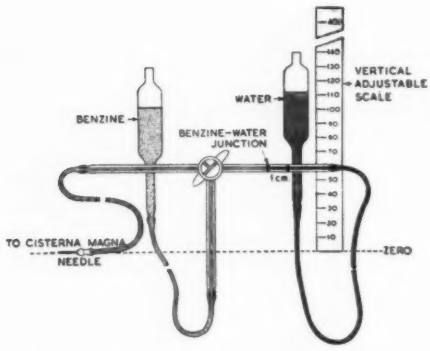

Fig. 1. Diagram of the manometer used for the determination of cisternal pressure.

The cerebrospinal fluid pressure was measured in a modified bubble manometer (Fig. 1). This apparatus consisted of a Pyrex glass T-piece of internal diameter 1 mm. Any two of the three arms of the T-piece could be joined by a glass tap which was lubricated by a fine suspension of bentonite in glycerine. One of the arms was connected by flexible Nylex tubing to a glass storage chamber, the second to a water-containing reservoir, and the third to the cisternal puncture needle. Two zero marks, 1 cm. apart, were placed on the arm leading to the water-containing reservoir.

In preparation for use, benzine was placed in the storage chamber and the whole apparatus filled from this source. Water was run into the other reservoir on top of the benzine to form a benzine-water junction, which was then run back into the arm of the T-piece leading to the water-containing reservoir until it reached the zero mark nearer the tap. The tap was rotated into a neutral position and the manometer was ready for use.

Prior to cisternal puncture, hair was removed from the rabbit's neck and ears, and blood was collected from an ear vein. The animal was then given, by intraperitoneal injection, 50 mg. of nembutal/kg. of body weight. This usually produced general anaesthesia with complete relaxation in about ten minutes and the cisternal puncture was performed within half an hour.

The measurement of the pressure and the collection of cerebrospinal fluid were carried out as follows. The anaesthetized animal was placed on its left side and a clear airway ensured. The external occipital protuberance, the posterior surface of the arch of the atlas and the spine of the axis vertebra were identified through the skin which was then cleaned with iodine. A small incision was made in the midline half way between the external occipital protuberance and the spine of the axis vertebra.

Meanwhile, the manometer was set up and the needle, boiled for 15 minutes, attached to the appropriate tube and filled with benzine. The head was held firmly in the left hand and the needle, without a stilette, was inserted through the skin incision into the muscles of the back of the neck. At this stage an assistant connected the needle through the tap to the water-containing reservoir, the benzine-water junction remaining, of course, at the first zero mark. The reservoir was lowered so that the water surface was slightly below the level of the cistern.

The needle was then passed immediately above the arch of the atlas vertebra into the cisternal space, the entry into which became easy to appreciate after some experience. In addition, the benzine-water junction immediately moved towards the second zero mark and away from the needle.

By raising and lowering the water-containing reservoir the assistant then adjusted the benzine-water junction to coincide with the second zero mark. There was a slight to-and-fro oscillation with the phases of respiration. The height of the column of water above the cistern was now a measure of the cisternal pressure and readings were made from a vertical adjustable scale. Readings could be repeated several times without altering the position of the needle.

The water-containing reservoir was then lowered and cerebrospinal fluid flowed out through the needle. Up to 1·5 ml. could be collected from a large rabbit (2–3 kg.) without danger.

RESULTS.

33 punctures have been performed on 28 normal rabbits. Five of these (Nos. III, 10a, 5, 16, 23) were punctured twice before treatment. The pressure and calcium concentration of the cerebrospinal fluid of the group are shown, together with some other data, in Table 1.

TABLE 1.

Results of 33 Cisternal punctures performed on normal rabbits.

Animal No.	Date	C.S.F. Pressure (mm H_2O)	C.S.F. Ca (mg. %)	Serum Ca (mg. %)	Wt. (gm.)	Remarks
III	(i) 2.2.54	—	6·4	—	1315	20 gauge needle. Turbid C.S.F.
	(ii) 13.4.54	94	6·6	—	2170	20 gauge needle
IV	2.2.54	68	5·5	—	765	20 gauge needle
V	9.2.54	76	5·4	—	2200	20 gauge needle
1a	10.9.54	93	—	—	1440	25 gauge needle. No C.S.F. collected
2a	14.9.54	81	6·6	13·2	2155	25 gauge needle. Turbid C.S.F.
3a	6.8.54	140	5·5	15·9	1680	25 gauge needle
4a	23.9.54	83	6·0	14·8	3200	25 gauge needle. Turbid C.S.F.
5a	16.7.54	116	5·6	15·4	2260	25 gauge needle
7a	13.7.54	121	6·5	15·8	1900	25 gauge needle. Turbid C.S.F.
8a	19.8.54	119	5·9	14·0	1550	25 gauge needle
10a	(i) 16.11.54	131	—	13·2	1955	25 gauge needle. No. C.S.F. collected
	(ii) 30.11.54	126	6·2	13·4	1950	25 gauge needle
2	1.6.54	95	5·7	—	3000	20 gauge needle
5	(i) 11.2.54	109	5·4	—	1750	20 gauge needle
	(ii) 14.4.54	—	5·8	—	1920	20 gauge needle
6	7.6.54	94	—	15·6	2900	20 gauge needle. C.S.F. contaminated
7	7.5.54	79	—	16·6	2980	20 gauge needle. Grossly blood-stained C.S.F.
11	22.6.54	121	6·2	—	2800	20 gauge needle
12	8.7.54	110	5·7	16·6	2400	25 gauge needle
13	1.12.54	100	5·6	14·6	2665	25 gauge needle
15	7.12.54	100	—	12·2	3055	25 gauge needle. No C.S.F. collected
16	(i) 20.1.55	116	5·4	12·5	2145	25 gauge needle
	(ii) 5.4.55	112	5·6	14·2	2410	25 gauge needle
17	20.1.55	109	5·2	13·6	2890	25 gauge needle
20	5.4.55	91	—	—	2465	25 gauge needle. No C.S.F. collected
21	3.5.55	110	4·9	13·6	2130	25 gauge needle
22	6.5.55	98	5·0	13·8	2120	25 gauge needle
23	(i) 9.5.55	122	5·0	12·2	2135	25 gauge needle. Turbid C.S.F.
	(ii) 16.5.55	101	5·1	13·4	—	25 gauge needle. Turbid C.S.F.
24	20.6.55	124	5·1	13·5	2550	25 gauge needle
27	29.7.55	113	5·0	13·5	2200	25 gauge needle
28	29.7.55	100	5·2	13·6	1720	25 gauge needle

Pressure readings were made 31 times on the 28 animals, i.e., the pressure was recorded in three rabbits (Nos. 10a, 16, 23) at separate punctures days or weeks apart. These 31 readings have a mean of 104·9 mm. of water and a range of 68–140 mm.

The calcium concentration of the cerebrospinal fluid of 23 rabbits was determined 27 times. (The mean of these values is 5·6 mg. p.c. with a range of 4·9–6·6 mg. p.c.) Suitable fluid was obtained at separate punctures from 4 animals (Nos. III, 5, 16, 23).

These 27 determinations were made on fluid which was perfectly clear or only slightly contaminated with red blood cells. In the instances where no value for calcium is included, no cerebrospinal fluid was obtained (Nos. 1a, 10a, 15, 20) or it was grossly blood-stained (No. 7). One specimen (No. 6) was collected into a bottle which was thought later to be contaminated.

If six samples which were noted at the time of puncture to be slightly turbid are excluded from the series, 21 values are left. These have a mean of 5·5 mg. p.c. and the same range as the whole group.

DISCUSSION.

The technique.

The cisternal puncture needles were made from 25 gauge "record" needles by breaking them off about one inch from the base and grinding a flat bevel. They had a rather blunt point but would pass with ease through the various structures encountered except skin, which was incised by a scalpel. Size 25 was quite satisfactory and there was less danger of fatal trauma to the brain-stem than from the larger 20 gauge needles which were used earlier.

Benzine is a fluid of low viscosity which is immiscible with water and a very suitable medium for use in the manometer. The benzine-water junction was convenient, sharp and clearly visible; but more important was the fact that the cerebrospinal fluid could be collected completely uncontaminated with water. Furthermore, there was no contamination from the air and the fluid could be stored, if desired, under paraffin.

An obvious disadvantage was the possibility of introducing benzine (an irritating fat solvent) into the subarachnoid space. This caused a number of deaths in the early experiments but was later avoided by placing two marks, 1 cm. apart, on the appropriate arm of the manometer. The benzine-water junction, initially at the mark nearer the needle, was finally adjusted to coincide with the more distal one and not allowed at any time to pass proximal to the first mark. In this way benzine was prevented from entering the cistern and there were no further deaths from this cause. Obviously, the technique measures the pressure of cerebrospinal fluid from which 8 $mm.^3$ (0·008 $cm.^3$) have been removed. This amount is insignificant relative to the total volume of the cerebrospinal fluid and the accuracy of the method.

50 mg. nembutal/kg. of body weight given intraperitoneally provided excellent anaesthesia within 10–15 minutes. Very occasionally severe respiratory depression developed but rarely until about 25 minutes after injection, by which time the puncture had been performed and fluid collected. Such animals always recovered after artificial respiration for several minutes. Careful attention was given to the airway and colour of the animal since anoxia and CO_2 retention are causes of raised intracranial tension. If there was any doubt about colour or airway, puncture was not performed, but such an event was extremely uncommon.

With due care and experience there are few risks and few losses, but the following points require attention. (i) Even a small amount of benzine in the subarachnoid space is lethal and must be prevented. (ii) Brain-stem trauma is a complication which can be avoided by using a fine, flat-bevelled needle and keeping it steady once the cistern has been entered; it is not a problem in experienced hands. (iii) Infection can be prevented by ordinary aseptic and antiseptic techniques, and we have not encountered this complication in any animal. (iv) Minor contamination of the cerebrospinal fluid with blood (presumably collected in the needle during insertion) did occur, though infrequently in the later experiments. This could possibly be reduced even further by using a stilette in the needle, but we do not think that this is desirable. The manipulation of removing a stilette complicates the task of holding the needle steady; also it prevents the benzine-water junction from indicating that the cistern has been entered. The smooth insertion of a hollow needle involving minimal trauma and entering the cistern at the first attempt will usually produce clear fluid.

With these precautions the technique is simple and safe. One rabbit has been punctured six times at intervals of about two weeks during prolonged hypocalcaemia; in others, three, four and five punctures have been performed without complications.

The results.

The cisternal punctures were performed at various times of the day and the animals were not fasted for any prolonged period beforehand except in the case of the last two. Consequently, no attempt has been made in this group of animals to correlate the calcium concentration of blood and cerebrospinal fluid.

The pressure was determined two or three times during most punctures and the figure given in the Table is the mean of these. In only one instance did the replicate readings vary by more than 5 mm. of water—viz., No. 11, in which the readings were 125, 115, 123 mm. of water.

Of particular interest are the three animals on which the pressure was determined twice at separate punctures before treatment began. The mean values in the case of Nos. 10a and 16 agree within 5 p.c.; but No. 23 had pressures of 122 and 101 mm. of water. This is a difference of about 20 p.c. and is

greater than that before and soon after treatment in any of the animals rendered hypocalcaemic (these results will be reported in detail later). Unfortunately there is no record of the weight of this animal on the day of the second puncture. Dehydration is well known to lower the cerebrospinal fluid pressure.

The calcium concentrations of the separate specimens obtained from four animals (Nos. III, 5, 16, 23) agree well. The six specimens which were noted to be slightly turbid have values ranging from 6·6 to 5·0 mg. p.c. This is substantially the same range as that of the whole group; nevertheless, if these six values are excluded, it reduces the mean value of the cerebrospinal fluid calcium concentration of the group by 0·1 mg. p.c.

SUMMARY.

A technique is described whereby the cerebrospinal fluid pressure can be measured and uncontaminated fluid collected from the rabbit.

The mean cerebrospinal fluid pressure and calcium concentration of 28 adult rabbits of both sexes were 104·9 mm. of water and 5·6 mg. p.c. respectively.

Some aspects of the technique are discussed and brief comment is made on the results.

Acknowledgments. Our thanks are extended to Miss P. Crowe, B.Sc., for her assistance during the cisternal punctures and with the animals generally. Thanks are also due to the Fairfax Institute of Pathology, Royal Prince Alfred Hospital, for supplying the animals, and to Miss B. James, Secretary.

REFERENCES.

Fales, F. W. (1953): J. biol. Chem., 204, p. 577.
Grant, D. K. (1953): Quart. J. Med., n.s. 22, p. 243.
Watson, L. C. A. (1956): Austral. J. exp. Biol., 34, p. 49.

THE EFFECT OF AMBIENT TEMPERATURE ON HAEMOGLOBIN CONCENTRATION

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(Accepted for publication 26th October, 1955.)

It has been observed that the mean haemoglobin values of comparable groups of both male and female subjects are significantly less in summer than in winter (Walsh, Arnold, Lancaster, Coote and Cotter, 1953). To explain these differences it was suggested that high ambient temperature during summer causes cutaneous vasodilation which produces an increase in the total volume of the vascular spaces. Fluids pass into the circulation from the intestines and from the interstitial fluids to increase the blood volume and thereby produce haemodilution. There is thus maintained a constant relationship between the volume of the blood vessel lumina and the volume of fluid which is contained within the vessels. This explanation of the observed seasonal differences is, however, hypothetical.

In this paper differences in haemoglobin values in female human volunteers have been specifically related to differences in the ambient temperature and attempts to reproduce the phenomenon in experimental animals are described.

MATERIALS AND METHODS.

Human volunteers. Venous blood was collected from 28 female subjects between 18 and 40 years of age on two different days. All were engaged in similar work on the two occasions and the samples were collected at 3.00 p.m. On the first day the temperature was 29·5° C. and the humidity 60 p.c. Three weeks later the second sample was obtained, the temperature then being 14·5° C. and the humidity 60 p.c. The blood samples were collected without application of a tourniquet to the arms and approximately 2·5 ml. of blood was mixed with one drop of heparin solution in each instance.

Experimental animals. Three different sets of experiments were performed with animals. In the first group large interbred mice which had been obtained from the Australian National University, Canberra, were used. Albino rats were used in the second series of animal experiments. They were subjected to splenectomy fourteen days before use and all were injected with neoarsphenamine (0·1 ml. of a 5 p.c. solution) immediately after splenectomy to control infection with *Bartonella muris*. For the third series special mice characterized by relative absence of hair were supplied by Dr. W. R. Sobey, of the Animal Genetics Section, C.S.I.R.O., University of Sydney. These animals of the naked (N) strain are a genetic variant and were used to investigate the effect of body hair as a factor influencing cutaneous vasodilation.

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[1]This work was undertaken during tenure of a Fellowship of the National Health and Medical Research Council.

Measurement of haemoglobin value. Blood for estimating the haemoglobin value was obtained from the tails of animals by cutting the tails and collecting the blood directly into a micro-pipette. When heart blood was used the mouse was anaesthetized with ether, the chest opened anteriorly and the heart incised. Blood was then pipetted from the chest cavity.

The technique of haemoglobin estimation with a photoelectric colorimeter has been described (Walsh *et al.*, 1953). Usually a dilution of 1 in 200 was prepared by adding 50 c.mm. of blood to 10 ml. of 0·2 p.c. ammonia solution. A 1 in 400 dilution was sometimes used with blood from the tails of mice and this was prepared by adding 25 c.mm. of blood to 10 ml. of ammonia solution.

Control of ambient temperature. Mice were placed under different temperature conditions as shown in the Tables. An incubator was used to obtain the temperatures of 30° C. and 37° C. There was a constant exchange of air in the cabinet, fresh air entering at the top of the cabinet and being evacuated from the lower portion. Drinking water was supplied during the experiment. The temperature of 6° C. was obtained in a cold room. The animals were not separated one from another in the first series of experiments but were placed in mouse boxes containing 10 or less mice to each box.

RESULTS.

Human volunteers. The haemoglobin values obtained on venous blood from 28 female subjects are shown in Table 1. The significance of the differences

TABLE 1.

Effect of Temperature on Haemoglobin Values of Venous Blood from Female Subjects on 2 different days.

	1st Day Temp. 29·5° C., Humidity 60 p.c.	2nd Day Temp. 14·5° C., Humidity 60 p.c.	Difference
1	12·3	13·4	+1·1
2	12·2	12·0	−0·2
3	13·0	13·9	+0·9
4	13·4	13·3	−0·1
5	13·4	14·3	+0·9
6	13·5	14·3	+0·8
7	11·3	12·4	+1·1
8	12·8	12·8	0
9	14·5	14·9	+0·4
10	13·2	13·2	0
11	14·5	14·7	+0·2
12	11·6	12·4	+0·8
13	12·2	12·3	+0·1
14	13·8	14·3	+0·5
15	12·3	12·7	+0·4
16	13·8	14·0	+0·2
17	12·1	12·1	0
18	13·1	13·5	+0·4
19	14·3	14·2	−0·1
20	12·8	13·8	+1·0
21	12·4	13·2	+0·8
22	13·8	14·6	+0·8
23	12·0	13·5	+1·5
24	13·0	12·8	−0·2
25	12·3	12·6	+0·3
26	14·3	14·3	0
27	14·2	14·3	+0·1
28	13·7	14·0	+0·3

has been tested by Fisher's t test. A value for t of 4·89 for 27 degrees of freedom was obtained. This gives P less than 0·001. Four of the subjects showed no change in the haemoglobin value, four a decrease, and the remaining 20 an increase on the colder day. This experiment was designed to reduce the number of possible variables. The activity of the subjects and the time of day when the tests were performed were the same on both occasions. Any effects of menstruation would have been equal on the two days. The effect of age has been eliminated by the use of the same subjects on the two occasions.

TABLE 2.

Mean Haemoglobin Values on Heart Blood of Mice under different conditions.

Treatment	Number of Animals	Mean Haemoglobin Value (grams)
37° C. for 1 hour	10	13·10
37° C. for 2 hours	10	13·37
37° C. for 4 hours	10	13·69
37° C. for 16 hours	8	13·26
37° C. for 20 hours	8	14·40
30° C. for 2 hours	20	13·59
30° C. for 4 hours	18	13·92
6° C. for 2 hours	19	13·52
6° C. for 20 hours	25	13·29
Total	128	13·55

Analysis of Variance.

Source of Variation	Degrees of Freedom	Sums of Squares	Variance
Between temperature conditions	8	13·1182	1·640
Within temperature conditions	119	99·9218	0·840
Total	127	113·0400	—

Variance ratio = 1·640/0·840 = 1·95 for $n_1 = 8$, $n_2 = 119$. This is not significant at the 5 p.c. point.

Experimental animals. Table 2 shows the results obtained on heart blood of mice after exposure to different ambient temperatures for varying periods of time. An analysis of variance shows that the mean values do not differ significantly. The highest mean value (14·04 grams) was obtained on mice kept at 37° C. for 20 hours and was probably the result of dehydration.

The values obtained on tail blood of some of the same mice are analysed in Table 3. The significant variance ratio is mainly due to the high mean value obtained on mice kept at 37° C. for 20 hours. This value is similar to that obtained for the heart blood of the same group of animals. Two mice in this group with haemoglobin values of 17·6 and 15·6 grams were partially

TABLE 3.

Mean Haemoglobin Values on Tail Blood of Mice under different conditions.

Temperature Conditions	No. of Mice	Mean Haemoglobin Value (grams)
37° C. for 1 hour	10	13·21
37° C. for 2 hours	9	13·79
37° C. for 4 hours	9	13·93
37° C. for 16 hours	7	13·70
37° C. for 20 hours	9	14·53
6° C. for 20 hours	27	12·88
Total	71	13·46

Analysis of Variance.

Source of Variation	Degrees of Freedom	Sums of Squares	Quotient (Mean Square)
Between Temperature conditions	5	23·5374	4·707
Within Temperature conditions	65	85·6046	1·317
Total	70	109·1420	—

Variance ratio = 4·707/1·317 = 3·57 for $n_1 = 5$, $n_2 = 65$. Significant at 1 p.c. level.

responsible for the large mean square of differences between the temperature conditions.

Two additional groups of mice were compared after exposure of one group to a temperature comparable to that experienced by the human volunteers on the colder day. The first group, comprising 15 animals, was held at 29° C. in a water saturated atmosphere for two and a half hours whilst the second group, of 14 animals, was placed in a refrigerator at 15° C. for the same time. The mean haemoglobin value of heart blood of the first group was 14·51±0·175 grams and of the second group 14·83±0·239 grams. The difference, 0·32±0·24 grams, is not significant.

The possible rôle of the spleen in determining changes in the haemoglobin value with variations in ambient temperature was investigated in rats. Two groups of splenectomized rats were used and tail blood tested. The first group of 15 animals was placed at 6° C. for two and a half hours and the haemoglobin values measured before and after this treatment. The ambient temperature immediately before the experiments was 21° C. One rat showed no change in the haemoglobin value, four a higher value after the period of low temperature exposure and the remaining 10 a lower level. The value obtained for t, 1·708 for 14 degrees of freedom, is not significant at the 5 p.c. level.

The second group of splenectomized rats, consisting of 14 animals, was exposed to a temperature of 37° C. for 2 hours. The ambient temperature of the laboratory was again 21° C. Seven animals showed an increased haemoglobin

value after exposure and the other seven a decreased value. Again the t test was applied but a significant value was not obtained (t=0·34 for 13 degrees of freedom).

The last animals tested were the mice with less than 50 p.c. of their body covered with hair. They were divided into two groups. The first consisted of 16 mice placed in the cold room at 6° C. for four hours. Each animal was placed in a glass beaker which was covered with wire gauze. At the end of four hours the haemoglobin value of the heart blood was measured. The animals of the second group of 18 mice were maintained at a temperature of 34° C. for four hours. Mean haemoglobin values of 15·07 grams for the group held at 6° C., and of 14·74 grams for the group at 34° C. were obtained. However, these values do not differ significantly.

DISCUSSION.

The results obtained on the human subjects show that ambient temperature influences the haemoglobin concentration of blood. This finding confirms the suggestion that seasonal differences in the values of comparable groups could be the result of temperature changes. Measurement of the blood flow in the skin of human subjects at different temperatures has shown that the higher the temperature the greater the blood flow. To prove the hypothesis advanced in the introduction to this paper, it remains to be shown that a high ambient temperature produces an increase in the plasma volume. However, the problem is not a simple one because direct plasma volume measurement is difficult. It is doubtful whether a sufficiently accurate technique is available to demonstrate the differences which would produce the relatively small changes that have been demonstrated in the haemoglobin concentration of the human subjects.

The experiments on mice show that these animals differ from the human in their reaction to environmental temperature. Stullken and Hiestand (1954) were also unable to demonstrate haematological changes in mice after short (15 minutes) or long (5 days) exposure to a temperature of 5° C. In the present work the possibility was considered that the spleen in rodents may be able to contribute proportionately a larger volume of concentrated red cells to the circulation than does the spleen of man. It seems unlikely that this is the explanation of the different results, because splenectomized rats behave in a manner similar to non-splenectomized mice. The presence of body hair also seems to be unimportant, because the relatively hairless mice failed to show haemodilution when exposed to a temperature of 37° C. for four hours.

There are at least two anatomical differences between man and the rodents which might be expected to have a bearing on physiological reactions to changes in the ambient temperature.

(1) The absence of hair and the presence of sweat glands in the skin of man facilitates the loss of heat from the body. On the other hand, the presence

of hair and the absence of sweat glands in rodents (Nay, T., personal communication) means that the skin is better designed to prevent than to allow heat loss.

(2) Surface area in relation to mass is much greater in rodents than in man. For this reason the problem in the rodent is to prevent excessive heat loss at low temperatures rather than to facilitate heat loss at higher temperatures. The insulating properties of the hair are possibly important.

In man, some adjustment of the skin vessels is apparently necessary to control heat loss, but this physiological adjusting mechanism is probably absent in the rodent. It may be developed only in man and be associated with the absence of hair. From the indirect evidence of haemoglobin concentrations, it appears to be absent from the variant "naked" mice.

SUMMARY.

The mean haemoglobin concentration of 28 human female subjects was found to be higher on a cold than on a hot day. The ambient temperature appeared to be responsible for the difference as all other variables were controlled. It is thought that a high temperature, by producing vasodilation of the cutaneous vessels, may increase the plasma volume with proportional haemodilution. Attempts to investigate the phenomenon in rodents were not successful because these animals do not react in the same manner to changes in ambient temperature. The difference in reactivity between man and rodents is not due to the greater size of the spleen or the presence of hair in the rodents.

Acknowledgments. The authors are indebted to Dr. H. O. Lancaster, of the School of Public Health and Tropical Medicine, University of Sydney, for assistance with the statistical analyses presented in the paper, and to Dr. F. C. Courtice, of the Kanematsu Institute, Sydney Hospital, for helpful advice. They are grateful to Dr. W. R. Sobey, of the Animal Genetics Section, C.S.I.R.O., University of Sydney, for supplying the "naked" mice.

REFERENCES.

Stullken, D. E. and Hiestand, W. A. (1954): Proc. Soc. exp. Biol., 86, p. 253.

Walsh, R. J., Arnold, B. J., Lancaster, H. O., Coote, M. A. and Cotter, H. (1953): A Study of Haemoglobin Values in New South Wales, N.H. and M.R.C. Special Report Series No. 5, Canberra.

WATER SOLUBLE COMPOUNDS (NON-KERATINS) ASSOCIATED WITH THE PLUMAGE OF THE PIGEON (COLUMBA LIVIA)

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(Accepted for publication 26th October, 1955.)

In establishing the concept of the non-keratins (water soluble non-protein organic constituents of keratinous structures) it became desirable to investigate at least one species of each class of vertebrates more fully. The pigeon was chosen as a representative of the class aves for its ready availability and the natural cleanliness of its plumage.

METHODS.

Pigeon feathers were obtained during the winter months from wild living birds. These were plucked immediately after they had been shot and only clean feathers were collected. As described previously (Bolliger and Gross, 1954), these feathers were washed with ether to remove the flakes associated with them, dried, weighed and extracted four times with boiling water for a period of approximately one hour each. The extracts were concentrated and solids contained in them divided into four main fractions, as described below.

Filter paper chromatography. Ascending and descending methods were used. Sugars and amino acids were run in water-saturated phenol containing a small amount of 8-hydroxyquinoline or in *n*-butanol-acetic acid-water (Partridge and Westall, 1948). The positions of the amino acids were detected by spraying with 0·2 p.c. ninhydrin in water-saturated *n*-butanol. Sugars were detected with aniline hydrogen phthalate (Partridge, 1949). Purines were developed in *n*-butanol-formic acid-water (Smith and Markham, 1950) or in *n*-butanol saturated with 10 p.c. urea (Carter, 1950) and detected by the mercuric nitrate method (Vischer and Chargaff, 1947).

Polypeptides were hydrolyzed with 8N sulphuric or hydrochloric acid for 24 hours. Carbohydrates were hydrolyzed in 0·5N sulphuric acid for 2 hours. Sulphuric acid was removed with barium carbonate and hydrochloric acid by repeated evaporation *in vacuo* prior to chromatographic analysis.

Displacement chromatography. The hydrolysate of the polypeptide fraction in 8N hydrochloric acid was adjusted to 1·5N and fractionated on a column of "Zeo-Karb" 225 (Wall, 1953). 5 ml. fractions were collected and examined by paper chromatography.

Quantitative methods were those used in previous papers (Bolliger and Gross, 1952, 1952a).

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[1]Working under a grant from the National Health and Medical Research Council.

RESULTS.

Pigeon feathers. 575 gm. of pigeon feathers were extracted with 18 litres of water and the total extract concentrated to 800 ml., yielding an amber coloured solution of pH 6·4.

Fraction A. On keeping the concentrate in the refrigerator overnight 1·3 gm. of crystals were obtained. Besides some calcium sulphate, this fraction contained mainly sparingly soluble organic substances. Uric acid and guanine were purified and examined spectrophotometrically; guanine, adenine, xanthine, hypoxanthine and tyrosine were identified by paper chromatography, and tyrosine by chemical tests. This fraction also gave a positive Sakaguchi test indicating the presence of guanidine compounds.

Fraction B. After removal of fraction A, 20 gm. of solid trichloracetic acid were dissolved in the solution and approximately 0·8 gm. of a polypeptide was precipitated. This precipitate was readily soluble in water. Hot glacial acetic acid was a convenient medium for purification in which the compound dissolved satisfactorily and settled out on cooling. On hydrolysis, the purified polypeptide yielded a number of amino acids. In order to facilitate identification by paper chromatography the hydrolysate was fractionated on a column of "Zeo-Karb" 225. The following sixteen amino acids were identified: aspartic and glutamic acids, serine, glycine, threonine, alanine, isoleucine(?), leucine, valine, proline, tyrosine, phenylalanine, arginine, histidine and lysine. Fraction B did not contain any purines or reducing substances before or after hydrolysis. Total nitrogen of the purified polypeptide was 13·6 p.c.

Fraction C. After removal of the trichloracetic acid, the aqueous extract was concentrated to 400 ml. and 9 volumes of absolute alcohol added. This precipitated fraction C (1·8 gm.) was redissolved in hot water. An insoluble portion (0·8 gm.) was found to be calcium sulphate with a small amount of calcium phosphate by chemical and X-ray spectrographic tests. The water-soluble part of fraction C was reprecipitated with alcohol. The resultant solid gave a reddish colour with iodine solution similar to that given by glycogen. On hydrolysis this fraction was found to contain glucose, xylose and arabinose. No reducing sugars were present before hydrolysis.

Fraction D. The remaining solution was concentrated *in vacuo* to about 15 ml. It contained, in a free state, all the amino acids found in the polypeptide, although cystine was present in traces only. Adenine, guanine, xanthine, hypoxanthine, glucose and ribose were also identified in this fraction without prior hydrolysis.

Pigeon flakes. 15 gm. of pigeon flakes were extracted and fractionated in the same manner. The results obtained were essentially the same as for pigeon feathers. Quantitative differences, however, were noted. The most striking of these was the higher amount of polypeptide (Table 1). The polysaccharide fraction gave a blue colour with iodine solution similar to that given by starch.

TABLE 1.

Quantitative Results of Pigeon Feathers and Flakes.

	Feathers	Flakes
Total solids	3·80 p.c.	9·63 p.c.
Total ash	0·40 p.c.	0·79 p.c.
Total N	324 mg. p.c.	790 mg. p.c.
Urea N	0 mg. p.c.	0 mg. p.c.
Ammonia N	60 mg. p.c.	95 mg. p.c.
Free amino acid N	20 mg. p.c.	44 mg. p.c.
Combined amino acid N	19 mg. p.c.	133 mg. p.c.
Uric acid (uricase method)	65 mg. p.c.	210 mg. p.c.
Total creatinine	24 mg. p.c.	76 mg. p.c.
Reducing substances*	280 mg. p.c.	647 mg. p.c.
Reducing substances hydrolysed	345 mg. p.c.	863 mg. p.c.
Pentose	102 mg. p.c.	288 mg. p.c.
Desoxyribose	35 mg. p.c.	—

* expressed as glucose.

DISCUSSION.

Materials.

In contrast to the feathers which are shed in their original form, the light blue powder released from them by organic solvents is composed of keratinous flakes of no definite structure. On a previous occasion (Bolliger and Gross, 1954) these were referred to as skin flakes in analogy with the skin flakes of mammals. However, certain birds, including the pigeon (columba livia), produce the so-called feather powder. This consists of finely divided keratinous particles and is furnished mainly by the disintegration of special down feathers. This powder is distributed over the plumage by the bird and has been said to serve as water repellant and lubricant (Pierre-P. Grassé, 1950). The material referred to in this paper as pigeon flakes consisted most probably predominantly of feather powder with an admixture of true skin flakes.

Nucleic acid derivatives.

As has been pointed out previously (Bolliger and Gross, 1952), keratinous structures are accompanied by by-products of keratinization, the non-keratins. A fundamental difference between the protein keratin and the epidermal cells from which it is derived is the absence of cell nuclei from the fully keratinized structure. This requires the disposal of considerable amounts of ribose and desoxyribose nucleic acids. According to quantitative and qualitative data on pigeon feathers and flakes, nucleic acid degradation products are present in large amounts in the non-keratin fraction of these keratinous materials. Adenine, guanine, xanthine, hypoxanthine, uric acid, ribose and desoxyribose could be detected and some of them were isolated. Of these guanine and uric acid occurred in larger amounts than any of the others.

Amino acids and polypeptide.

Extracts of pigeon feathers and flakes contained considerable quantities of a polypeptide which showed a similar qualitative amino acid composition to feather keratin (Graham *et al.*, 1949; and personal observations). This polypeptide may represent a fraction of the feather or flake keratin which is incompletely keratinized and therefore soluble in water, or it may constitute cellular material which was not used for inclusion into the keratin molecule. In view of the recent work on water soluble proteins which may be extracted from wool with alkaline thioglycollate (Gillespie and Lennox, 1953), it is noteworthy that feathers contain a directly water-extractable polypeptide or protein. In this paper the term "non-keratins" includes water-soluble proteins as well as other water-soluble substances.

The free amino acids identified in pigeon feather and flake extracts are at present considered as precursors or by-products of keratinization. They were identical with those found in the water-soluble polypeptide although cystine was only present in traces. So far free cystine has not been detected in fur or hair extracts (Rothman, 1954), or skin flakes (Bolliger and Gross, 1954a); nor was it found among the amino acids of skin wipings (Rothman and Sullivan, 1949). The significance of the absence of cystine has been emphasized with reference to keratinization which is thought to be preceded by hydrolysis of epithelial proteins and incorporation of sulphur containing fragments *in statu nascendi* (Rothman, 1954). The very small amount of free cystine found in feather keratins would not invalidate this theory, more especially since it may arise from partial breakdown of the polypeptide during extraction and concentration.

Reducing sugars and polysaccharides.

Free glucose and ribose were found in fraction D. Fraction C contained one or more polysaccharides which could be precipitated with alcohol and gave a colour with iodine which in some preparations, especially those from pigeon flakes, was pure blue as with starch, and in feathers more reddish as with glycogen. The polysaccharide fraction always yielded xylose and arabinose in addition to glucose after hydrolysis. Whether the pentoses are combined with glucose or attached to some other radical is not known, though soluble carbohydrates associated with cellulose and containing glucose, arabinose and xylose have already been mentioned (Huffman *et al.*, 1955).

Inorganic salts.

Although organic sulphur in any appreciable amount only occurred in the polypeptide, inorganic sulphur in the form of calcium sulphate was present in considerable quantity. Determinations of inorganic sulphur associated with non-keratins of various sources should give valuable information.

The low inorganic phosphate content of extracts of keratinous materials presents a puzzling feature in view of the presence of considerable amounts of other nucleic acid derivatives such as pentoses and purines.

SUMMARY.

Aqueous extracts of pigeon feathers were examined. They contained a readily water-soluble polypeptide of similar amino acid composition to the insoluble keratin; polysaccharides containing glucose, arabinose and xylose; sixteen free amino acids; adenine, guanine, xanthine, hypoxanthine and uric acid; free glucose and ribose, as well as inorganic sulphates and phosphates.

Organic solvents released a pale blue powder from pigeon feathers which consisted of keratinous flakes. Aqueous extracts of these flakes were of similar qualitative composition to those of feathers, although the amounts of water-soluble constituents were much higher in flakes.

REFERENCES.

Bolliger, A. and Gross, R. (1952): Austral. J. exp. Biol., 30, p. 181.
Bolliger, A. and Gross, R. (1952a): *Ibid.*, p. 399.
Bolliger, A. and Gross, R. (1954): *Ibid.*, 32, p. 747.
Bolliger, A. and Gross, R. (1954a): Austral. J. Sci., 16, p. 190.
Carter, C. E. (1950): J. Amer. Chem. Soc., 72, p. 1466.
Gillespie, J. M. and Lennox, F. G. (1953): Biochem. biophys. Acta, 12, p. 481.
Graham, C. E., Waitkoff, H. K. and Hier, S. W. (1949): J. biol. Chem., 177, p. 529.
Grassé, Pierre-P. (1950): Traité de Zoologie, Vol. 15, Masson et Cie., Paris.
Huffman, C. W., Rebers, P. A., Spriestersbach, D. R. and Smith, F. (1955): Nature, 175, p. 990.
Partridge, S. M. and Westall, R. G. (1948): Biochem. J., 42, p. 238.
Partridge, S. M. (1949): Nature, 164, p. 443.
Rothman, S. (1954): Physiology and Biochemistry of the Skin, University of Chicago Press, Chicago, p. 372.
Rothman, S. and Sullivan, M. B. (1949): J. invest. Dermat., 13, p. 319.
Smith, J. D. and Markham, R. (1950): Biochem. J., 46, p. 509.
Vischer, E. and Chargaff, E. (1947): J. biol. Chem., 168, p. 781.
Wall, J. S. (1953): Analytical Chem., 25, p. 990.

SEROLOGICAL GROUP REACTIONS OF MURRAY VALLEY ENCEPHALITIS VIRUS[1]

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(Accepted for publication 21st June, 1955.)

Murray Valley encephalitis (MVE) has all the epidemiological characteristics of an arthropod-borne virus disease (Anderson *et al.*, 1952). Neutralization tests performed by Paterson *et al.* (1952) showed that three strains of MVE virus were related to, but antigenically distinct from, 13 strains of Japanese B encephalitis virus. In complement-fixation tests reported by Sabin (1952), a serological relationship was demonstrated between Japanese B, West Nile, St. Louis, yellow fever, and dengue viruses, but no crossing was demonstrated between Japanese B mouse antiserum and Western equine encephalitis or Rift Valley fever viruses. Haemagglutination-inhibition tests performed by Casals and Brown (1954) showed that the arthropod-borne viruses formed two serologically distinct groups, A and B. Group A included the equine encephalitides, eastern (EEE), western (WEE), and Venezuelan (VEE), and Sindbis and Semliki Forest viruses. Group B included the human encephalitides, Japanese B (JBE), St. Louis (SLE), and Russian spring summer (RSSE), and the tropical viruses, yellow fever (YF), dengue types 1 and 2, West Nile (WN), Ilhéus, Ntaya, Uganda S., and Zika. Within each group serological cross-reactions occurred between each member.

This paper presents evidence to show that MVE virus behaves as a typical group B member of the arthropod-borne viruses on the basis of haemagglutination-inhibition and complement-fixation tests.

Methods and Materials.

Antigens. MVE virus antigen was prepared from the brains of infected suckling mice by the acetone-ether extraction method of Casals *et al.* (1951). The method consisted essentially of the removal of lipides by five successive treatments, first with acetone and then with ether. A saline extract of the dried material was then made, and, after centrifugation at 9000 *g.*, the clear supernatant was used as antigen. The same antigen was used both in haemagglutination and complement-fixation tests. Antigens of other arthropod-borne viruses prepared by the same method were generously supplied by Dr. J. Casals, of the Rockefeller Foundation.

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[1]This work was performed during the tenure of a Rockefeller Foundation Fellowship.

Sera were prepared by repeated intraperitoneal injection of MVE virus into mice on five separate occasions, or by a single intracerebral injection of guinea-pigs. Prior to use in the haemagglutination-inhibition test, all sera were treated with Bentonite (USP) to remove non-specific inhibitors, and subsequently, with newly-hatched chicken erythrocytes to remove non-specific agglutinins. This method has been described by Clarke and Casals (1955). Dr. Casals kindly supplied antisera prepared against other arthropod-borne viruses.

Diluents. Dilutions of antigens and sera were made in a 0·05 M borate 0·12 M NaCl solution at pH 9·0. All manipulations were performed in an ice bath at 1–2° C. The "virus-adjusting diluents" for the chicken erythrocytes were a series of 0·2 M phosphate—0·15 M NaCl solutions (Clarke and Casals, 1955) which, when added in equal quantity to the antigen-serum mixture at pH 9·0, produced a series of pH values between 6·4 and 7·0.

Erythrocytes. Blood obtained from one-day-old chickens was added to Alsever's solution in the proportion 1:4 and stored at 4° C. (Chanock and Sabin, 1953). Immediately prior to use, the cells were washed three times in physiological saline, and the optical density of a 0·25 p.c. suspension, prepared from the stock 10 p.c. suspension, was determined. A cell suspension calculated to give an optical density of 0·500 at 490 mμ was prepared in the appropriate adjusting diluent (Clarke and Casals, 1955).

Haemagglutination tests.

(a) *Titration of antigen.* Serial twofold dilutions of 0·5 ml. of MVE antigen were made in borate-saline solution at pH 9·0. 0·5 ml. of a suspension of chicken erythrocytes at optical density 0·500 in a virus-adjusting diluent to give pH 6·4 were added, and the cell pattern was read after two hours' incubation at 37° C. The usual haemagglutination titre under these conditions was 1/3200 per 0·5 ml. or 1/1600 per 0·25 ml.

Other antigens were titrated similarly, but the pH was varied to provide optimal titre for each virus. Where possible, mixtures of cells with serum and virus were incubated at 37° C., but some mixtures, particularly those involving RSSE, were incubated at 4° C. for two hours.

(b) *Titration of sera.* Serial twofold dilutions of bentonite-treated, cell-absorbed serum were made in borate-saline solution pH 9·0, and 0·25 ml. aliquots of each dilution were distributed in tubes. To each tube were added 8 agglutinating doses (A.D.) of antigen in a volume of 0·25 ml. at pH 9·0, and the mixtures were held overnight at 1 to 2° C. Chicken erythrocytes suspended in the appropriate virus-adjusting diluent were added the following morning, and the pattern of cell agglutination was read after two hours' incubation at 37° C. or 4° C. The titre of the serum was taken as the reciprocal of the highest dilution which caused complete inhibition of haemagglutination by 8 A.D. of antigen.

Complement-fixation tests. All sera were heated at 60° C. for 20 minutes prior to use in the test. The sheep erythrocytes, haemolysin and complement which were used in the test were all obtained commercially. Freshly prepared physiological saline was always used.

"Box" titrations of each antigen against each serum were performed according to the method of Casals *et al.* (1951). The complement was titrated immediately prior to use and 1½ complete haemolytic units were added. Mixtures of antigen, complement, and serum were held overnight at 4° C. Sensitized sheep cells were added, and the reactions were read after 30 minutes at 37° C.

RESULTS.

Haemagglutination-inhibition tests. Sera prepared against MVE and JBE viruses were tested against the antigens of MVE, JBE, SLE, RSSE, YF, Ilhéus, and Dengue Type 1 viruses. Incubations of serum-virus mixtures and chicken

erythrocytes were carried out at 37° C. on one occasion and at 4° C. on another.

Table 1 shows the extensive serological cross-reactions which occurred between sera prepared against MVE and JBE viruses and the haemagglutinins of MVE, JBE and SLE. There was a definite but somewhat lower titre of cross-reactions between MVE antisera and RSSE, YF, Ilhéus, and Dengue Type 1 viruses. In general, cross-reactions occurred at a lower titre when haemagglutinins were tested against MVE antiserum prepared in the guinea-pig than in the mouse.

TABLE 1.

Inhibition of Haemagglutination by MVE and JBE Antisera

Antigen	Antisera			
	Incubation at 37°C		Incubation at 4°C	
	anti-MVE (mouse)	anti-MVE (guinea pig)	anti-MVE (mouse)	anti-JBE (mouse)
MVE	640	320	160*	320*
JBE	160	40	640	>5120
SLE	160	40	320	640
RSSE	—	—	80	80
YF	40	10	20	—
Ilhéus	160	40	40	—
Dengue Type 1	—	—	20	—

Titres expressed as the reciprocal of the highest dilution of serum which caused complete inhibition of haemagglutination by 8 A.D. of antigen.

* Incubated at 37°C for 2 hours after addition of chicken erythrocytes.

— Not tested.

Mouse sera prepared against other arthropod-borne viruses were tested against MVE antigen. Sera prepared against the following group viruses JBE, SLE, WN, RSSE, YF, Dengue Type 2, Uganda S, Zika and Ntaya gave significant inhibition of haemagglutination by MVE virus. No inhibition was shown by antisera for three group A viruses (EEE, WEE, VEE).

Complement-fixation tests. Complement-fixation reactions of the "box" type were performed between several antigens and mouse sera prepared against MVE and other arthropod-borne viruses. The optimal titre of MVE antigen was usually found to be 0·1 ml. of a 1/32 dilution. The titre of serum was recorded as the reciprocal of the highest dilution with which 1½ complete haemolytic units of complement were fixed in the presence of optimal titre of antigen.

Table 2 presents the results of the complement-fixation tests. MVE antigen reacted strongly with mouse sera prepared against MVE, JBE, WN, and Ilhéus at titres between 32 and 128. A weak reaction was recorded with yellow fever antiserum (upper row). MVE mouse antiserum reacted with the antigens of MVE, JBE and SLE viruses, but not with other group B antigens, and there was no reaction with group A antigens (lower row).

TABLE 2.

Complement-Fixation Reactions of Some Arthropod-Borne Viruses

	MVE	JBE	SLE	WN	YF	Ilhéus	Dengue Type 1	Dengue Type 2	RSSE	EEE	WEE
Titre of above serum against MVE antigen	32*	64	—	128	4	32	—	—	—	—	—
Titre of anti-MVE serum against above antigen	32	8	8	0	0	0	0	0	0	0	0

* Reciprocal of highest dilution of serum with which complement was fixed in the presence of optimal amount of antigen.
— Not tested.

DISCUSSION.

The serological reactions of MVE virus when examined by the haemagglutination-inhibition and complement-fixation tests have demonstrated a close relationship between this virus and other arthropod-borne viruses of Casals' group B, such as JBE, SLE and YF. This relationship was to be expected because of the similarity in epidemiological features (Burnet, 1952), host range (French, 1952), and haemagglutinin production (Macdonald, 1952) between MVE and other arthropod-borne viruses. A formerly obscure serological relationship between yellow fever and other tropical viruses such as dengue and Ilhéus, on the one hand, and the human encephalitides of the temperate zone, SLE and JBE, on the other hand, was made clear by the work of Casals and Brown (1954). MVE virus bears the same relationship to the various tropical arthropod-borne viruses as SLE and JBE.

Concerning the usefulness of haemagglutination techniques as a means of investigating the epidemiology of arthropod-borne virus infections, studies in progress at the Rockefeller Foundation Virus Laboratories have shown that sera from human beings convalescent from yellow fever or dengue reacted with virus antigens other than the one responsible for the illness (Casals, personal communication). If, however, it is reasonably certain that only one virus has affected a community, both in the past and in the particular season under consideration, as, for example, MVE virus in south-eastern Australia, haemagglutination-inhibition techniques may provide a most useful tool for the diagnosis of human cases and investigation of the spread of disease in the community.

SUMMARY.

MVE virus gave haemagglutination and complement-fixation reactions typical of group B members of the arthropod-borne viruses.

The possible use of haemagglutination techniques in epidemiological investigations is discussed.

Acknowledgment. I am indebted to Dr. J. Casals for much helpful advice during the course of this work.

REFERENCES.

Anderson, S. G., Donnelley, M., Stevenson, W. J., Caldwell, N. J. and Eagle, M. (1952): Med. J. Austral., 1, p. 97.

Burnet, F. M. (1952): Amer. J. pub. Health, 42, p. 1519.

Casals, J. and Brown, L. V. (1954): J. exp. Med., 99, p. 429.

Casals, J., Olitsky, P. K. and Anslow, R. O. (1951): *Ibid.*, 94, p. 123.

Chanock, R. M. and Sabin, A. B. (1953): J. Immunol., 70, p. 271.

Clarke, D. H. and Casals, J. (1955): Proc. Soc. exp. Biol. Med., 88, p. 96.

French, E. L. (1952): Med. J. Austral., 1, p. 100.

Macdonald, F. (1952): Brit. J. exp. Path., 33, p. 537.

Paterson, P. Y., Ley, H. L., Wisseman, C. L., Jr., Pond, W. L., Smadel, J. E., Diercks, F. H., Hetherington, H. D. G., Sneath, P. H. A., Witherington, D. H. and Lancaster, W. E. (1952): Amer. J. Hyg., 56, p. 320.

Sabin, A. B. (1952): In" Viral and Rickettsial Infections of Man," T. M. Rivers, ed. (2nd ed.; Philadelphia: J. B. Lippincott Co.), p. 556.